Gynodioecy in Mountain Thyme (*Thymus praecox* agg.): Why More Females at Higher Altitudes?

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von

Urs Landergott

von

Winterthur ZH

Promotionskomitee

Prof. Dr. H. Peter Linder (Vorsitz)
Prof. Dr. J. Jakob Schneller und Dr. Rolf Holderegger (Leitung der Dissertation)
Prof. Dr. Elena Conti
Prof. Dr. David E. McCauley
Dr. Jacqui A. Shykoff

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General introduction

Imagine you were visiting an alpine botanical and experimental garden situated in the vicinity of a famous tourist resort in the Swiss Alps. Right next to the collection of alpine plant species, you see a large purple patch consisting of many individuals of a creeping thyme species that immediately attracts the arriving visitors. As you get closer, you see small hut-like cages scattered across the field, and somebody lying in the middle of the purple carpet, manipulating flowers, further arousing the visitors' curiosity: "Excuse me, may I ask you what you're doing here with all this thyme? It's all the same, isn't it?" I used to draw the visitors' attention to the fact that actually not all the plants in the field were quite the same: there were individuals with large, perfect flowers with both male and female reproductive organs (hermaphrodites), but there were also individuals with smaller flowers which had no or severely reduced male organs and were thus completely male-sterile (females). I would further explain that my hand-pollination experiment was trying to understand the coexistence of females and hermaphrodites (referred to as gynodioecy; Darwin, 1877). Visitors involved in such a discussion often readily responded to the evolutionary focus: "There are purely female plants? That's interesting, because from what I've observed in my garden I would have thought that all plants were hermaphrodite!" Indeed, the majority of flowering plant species are hermaphrodite (Richards, 1997), but the transition from hermaphroditism to polymorphic systems with unisexual plants is a repeated trend in floral evolution, and gynodioecy has evolved in different plant families (Geber *et al.*, 1999). "And there are no purely male plants in thyme?" In fact, gynodioecy also is of interest as a transition stage in the evolution of dioecy, the separation of sexes onto male and female plants (Geber *et al.*, 1999). However, in several taxonomic groups for example, the Lamiaceae, gynodioecy appears to be a stable breeding system, since dioecy is rare in these groups (Darwin, 1877; Geber *et al.*, 1999). Moreover, in *Thymus*, hermaphrodites have been shown to retain significant female function, thus raising the question of the maintenance of females (Manicacci *et al.*, 1998; Thompson *et al.*, 1998). "But how do you address this question? Why did you choose Mountain Thyme for your investigations, apart from taking the opportunity to enjoy such a nice workplace?" Working and living in an alpine environment is actually not always as favourable as under the conditions that attract tourists! But it is precisely the increase in habitat harshness along elevation gradients that renders mountain ranges well suited for hypothesis testing in comparative evolutionary ecological studies (Körner, 1999). Mountain Thyme is widely distributed in the Swiss Alps from subalpine to alpine altitudes, and the frequency of females in populations increases with increasing altitude (Chapter 1). At this point, some people – mostly the women – would say: "So there is an obvious explanation for the existence of females: they are more robust than the hermaphrodites!" This hypothesis picks up the basic reasoning behind my studies on Mountain Thyme, that is, searching for an explanation for the occurrence of more females at higher altitudes in order to understand the maintenance of the gynodioecious system.

Background

The coexistence of female and hermaphrodite individuals within plant populations has attracted a great deal of interest ever since Darwin's (1877) publication on the *The Different Forms of Flowers on Plants of the Same Species* (Geber *et al.*, 1999). Extensive studies on the inheritance of sex have revealed that the two sexual phenotypes are determined by complex interactions between nuclear and cytoplasmic genes in most gynodioecious plant species (e.g. *Thymus*; Charlesworth & Laporte, 1998). Thus, there is a complex genetic polymorphism that needs to be maintained in order for the two sexual phenotypes to be maintained within a population. This challenges empirical research attempting to identify the evolutionary forces involved in the maintenance of nuclear-cytoplasmic gynodioecy (Charlesworth, 1999; Jacobs & Wade, 2003).

The male-sterility of females is caused by cytoplasmic genes (mitochondrial mutants), which suppress the male function (Budar *et al.*, 2003). Cytoplasmic male-sterility (CMS) genes are maternally inherited (via seeds) and can invade hermaphrodite populations if they are coupled with a female advantage in seed fitness, i.e. if females produce more or better quality seeds than hermaphrodites (Charlesworth, 1999). Several different CMS genes are generally found in natural populations (Charlesworth & Laporte, 1998). The hermaphrodites in gynodioecious populations typically also carry a CMS gene, but in combination with nuclear genes (transmitted to the next generation via pollen *and* seed) that had evolved to restore the male function when in combination with a particular CMS gene (Charlesworth & Laporte, 1998; Jacobs & Wade, 2003). Hence, both the nuclear and the cytoplasmic sex-determining genes may be present in both sexual phenotypes, and the specific interactions between particular nuclear-cytoplasmic combinations determine the sexual phenotype of individual plants.

Hermaphrodites depend on the presence of restorer genes that are appropriate to restore their particular CMS type. As a consequence, non-equilibrium dynamics between nuclear and cytoplasmic sex-determining genes and factors affecting the genetic diversity at sex-determining loci may cause sex ratio variation that is largely independent of ecological factors influencing the relative seed fitness of the two sexual phenotypes (Frank & Barr, 2001). In the extreme case, sex ratio variation may simply reflect different phases of limit cycles in a dynamic equilibrium of sex-determining genes, without invoking any ecological cause (Gouyon *et al.*, 1991). The consistent correlation between elevation and sex ratios in Mountain Thyme though suggests that among population variation in sex ratios is not attributable to limit cycles in this species (Chapter 1). However, factors affecting the genetic diversity at sex-determining loci, e.g. random genetic drift (Frank & Barr, 2001), may still cause a sex ratio gradient if their magnitude is correlated with an ecological gradient. The Mediterranean *T. vulgaris*, a species showing extraordinarily high and variable frequencies of females, provides a well-studied example of the effects of reduced genetic diversity on sex ratios (Thompson, 2002). This early-successional species has patch-structured metapopulations and rapidly colonises disturbed patches. Females attain highest frequencies in youngest patches of

T. vulgaris due to a temporary lack of appropriate restorer alleles during population establishment (founder effect; Thompson, 2002). This example highlights the importance of taking into account genetic diversity at sex-determining loci when studying sex ratio variation in species with nuclear-cytoplasmic gynodioecy.

However, the nuclear and cytoplasmic sex-determining genes are also exposed to the environment in the form of the sexual phenotypes, which will transmit the genes to the next generation. Because offspring of hermaphrodite seed-parents tends to be hermaphrodite-biased and offspring of females tends to be female-biased under nuclear-cytoplasmic sex-determination, ecological conditions altering the relative seed fitness (the contribution to the next generation via seeds) of the two sexual phenotypes may also influence equilibrium sex ratios (Charlesworth, 1999). For instance, pollen limitation, if affecting the seed set of females more severely than that of hermaphrodites, will reduce the relative contribution of females to the next generation and, consequently, influence population sex ratios (Maurice & Fleming, 1995; Olson *et al.*, 2005). Indeed, females and their offspring have often been shown to outperform hermaphrodites and their offspring in several fitness traits, such as flower production, seed production, seed weight, germination success and seedling survival (female fertility advantage; Darwin, 1877; Shykoff *et al.*, 2003), providing templates for environment-dependent selection to alter the relative seed fitness of the two sexual phenotypes. A positive correlation between the magnitude of a female advantage and the female frequency among populations would indicate a role of female advantage in causing sex ratio variation (Delph & Carroll, 2001). The proximate cause of such a female advantage may thus point to the mechanisms involved in the maintenance of gynodioecious systems (Charlesworth, 1999; Webb, 1999).

Female advantage may result from different genetic causes, which need to be isolated in order to understand the evolutionary processes involved in the maintenance of nuclear-cytoplasmic sex systems (Bailey *et al.*, 2003; Jacobs & Wade, 2003). For instance, outcrossed seed-offspring from hermaphrodites often perform less well than those from females (Gigord *et al.*, 1999; Delph & Mutikainen, 2003). This female advantage may result from the reallocation of resources that hermaphrodites devote to the male function (Darwin, 1877), potentially governed by positive pleiotropic fitness effects of CMS genes (Budar *et al.*, 2003). Alternatively, female advantage may result from negative pleiotropic fitness effects associated with the nuclear restorer genes, which will on average affect hermaphrodites more severely than females since hermaphrodites, on average, carry more restorers than the females (Delph & Mutikainen, 2003). Such negative pleiotropic effects of restorers (cost of restoration of male function) are predicted from evolutionary theory, because, without negative pleiotropic effects, restorer alleles would drive to fixation (i.e. all individuals carrying the corresponding CMS gene would be hermaphrodite; Bailey *et al.*, 2003). As a consequence, frequency- or genomic context-dependent fitness of restorer alleles, or heterozygote advantage at restorer loci may cause balancing selection to maintain nuclear-cytoplasmic polymorphism (Gigord *et al.*, 1999; Jacobs & Wade, 2003; Charlesworth, 2006). These considerations, though simplified (Gigord

et al., 1999; Bailey *et al.*, 2003; Jacobs & Wade, 2003), illustrate how the nuclear-cytoplasmic genetic constitution of individuals may also affect their seed fitness and survival, and they emphasise the importance of understanding and distinguishing the various selective forces acting upon nuclear-cytoplasmic sex systems.

The avoidance of inbreeding by obligately outcrossed females provides a further possible cause of female advantage (outcrossing advantage) that has often been supposed to contribute to the maintenance of females in gynodioecious populations (Mather, 1940; Delph, 1990; Webb, 1999). An outcrossing advantage of females requires significant self-fertilisation of hermaphrodites and reduced fitness of selfed *vs.* outcrossed offspring (costs of self-fertilisation). Both components have been detected in several gynodioecious species (Webb, 1999). However, empirical studies that estimate the magnitude of outcrossing advantage under natural conditions and relate the resulting female advantage to sex ratio variation among populations remain scarce (Webb, 1999; Medrano *et al.*, 2005). In Mountain Thyme, selfing rates and/or costs of selfing that would increase with increasing elevation could cause a positive correlation between female frequency and outcrossing advantage and, consequently, explain the sex ratio variation across altitudes. Intriguingly, this scenario parallels hypotheses that shifts in pollination biology (e.g. different pollinator guilds, decreased pollinator efficacy) may lead to enhanced pollinator-mediated geitonogamous selfing at higher altitudes (Delph, 1990) and that harsh alpine conditions may intensify the expression of costs of selfing (von Arx *et al.*, 2006).

Outline of the thesis

Mountain Thyme, *Thymus praecox* agg. Opiz ampl. Jalas (Lamiaceae), shows enormous morphological diversity that led taxonomists to describe a wealth of subspecies, varieties and forms from the European Alps. However, different individuals within populations can often be assigned to different *T. praecox* taxa, and characters of a given individual do often not allow unambiguous assignment to any of these taxa, suggesting random recombination between morphotypes. This high morphological diversity is the likely result of polyploidization and hybridisation in the evolutionary history of Mountain Thyme. The species is thus best treated as a single tetraploid aggregate (Jalas, 1970). Polyploidy implies that the nuclear genome became multiplied, which causes difficulties for molecular genetic analyses (Chapter 2), but may also cause pronounced effects of potential costs of nuclear restorer genes in *T. praecox*. Additional details on the biology of the study species are given in the three chapters of this thesis.

Chapter 1 examines the diversity and spatial distribution of sex-determining genes in Mountain Thyme, as inferred from sex ratio data. Corresponding questions are: (1) Is the observed sex ratio variation along elevation gradients attributable to reduced diversity at sex-determining loci at higher altitudes? (2) Is there evidence of natural selection affecting the local frequency of sex-determining alleles? (3) How could variation in the relative contribution

of seed-offspring of the two sexual phenotypes at contrasting altitudes contribute to the observed sex ratio variation among adult populations?

Females displayed a substantial female fertility advantage both in terms of quantity and quality of seeds as compared with hermaphrodites in natural populations of Mountain Thyme (number of seeds, seed weight, germination success; Landergott, unpublished data). However, there was no evidence of a causal relationship between female fertility advantage and sex ratio variation across altitudes. On the contrary, the relative advantage of females in the total number of produced seeds was slightly reduced at higher altitudes, possibly due to increased pollen limitation of females. This stresses the importance of further investigating the relative quality of the offspring of the two sexual phenotypes. The next two chapters of this thesis therefore focus on the outcrossing advantage of females.

Chapter 2 describes the technical development of molecular genetic (microsatellite) markers applicable to estimate outcrossing advantage in natural populations of tetraploid Mountain Thyme. While this chapter also gives insight into the reticulate evolutionary history of the study species, it most notably illustrates how molecular genetic markers can provide reliable estimates of heterozygosity in polyploid species with polysomic inheritance, which have hitherto been severely underrepresented in population genetic studies of plants (Soltis *et al.*, 2004).

Chapter 3 addresses the question of whether the outcrossing advantage of females maintains sex ratio variation across elevation gradients in Mountain Thyme. The microsatellite markers are used to examine the two major components of outcrossing advantage: namely rates and costs of self-fertilisation of hermaphrodites in low *vs.* high altitudinal natural populations. Corresponding questions are: (1) Do rates of geitonogamous self-fertilisation of hermaphrodites point to a shift in pollinator behaviour, which promotes increased pollinator-mediated selfing at alpine sites? (2) What are the major determinants of selfing rate in Mountain Thyme, and do hermaphrodites from high altitude populations experience higher selfing rates than those from low altitude populations? (3) Do selfed offspring have a lower chance to reach reproductive maturity at alpine sites than at subalpine sites?

In the concluding summary, I provide a synopsis of the findings of this thesis as well as perspectives for future research.

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Chapter 1

Sex ratio variation and spatial distribution of nuclear and cytoplasmic sex-determining genes in gynodioecious *Thymus praecox* agg.: why more females at higher altitudes?

Urs Landergott, J. Jakob Schneller, Rolf Holderegger & John D. Thompson

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Abstract

The evolution of a persistent polymorphism for both cytoplasmic male-sterility (CMS) genes and nuclear restorers of male function is a prerequisite for the maintenance of gynodioecy (co-existence of hermaphrodites and females) in many plant species. We used sex ratio data to explore the spatial variation of sex-determining genes and the proximate causes of sex ratio variation in gynodioecious *Thymus praecox* agg. The proportion of hermaphrodites in adult populations decreased with increasing altitude. Progeny sex ratios from natural populations and from controlled crosses within and among populations indicated that diversity at sex-determining loci was similar at contrasting altitudes and that certain alleles were widely distributed. Spatial correlations among sex-determining alleles across populations provided evidence of selection affecting local frequencies of CMS-restorer combinations. Estimates of the relative seed fitness of the two sex types, inferred from comparisons of adult and offspring sex ratios, suggested that environment-dependent selection governs sex ratio variation across altitudinal gradients in this species.

Keywords: cost of restoration; cytonuclear interactions; elevation gradients; genetic diversity; gender dimorphism; local adaptation; nuclear-cytoplasmic gynodioecy; sex expression; sex ratio

Introduction

The maintenance of gynodioecy, a form of gender dimorphism with both female and hermaphrodite individuals (Darwin, 1877), depends on a persistent polymorphism at both cytoplasmic and nuclear sex-determining loci in many plant species (Charlesworth, 1981; Bailey *et al.*, 2003). Understanding the frequencies of the two sexual phenotypes thus requires knowledge on the evolutionary dynamics of sex-determining alleles (Frank & Barr, 2001). The various selective forces acting upon the sex-determining genes depend, in turn, on phenotypic background and the differing modes of gamete transmission in the two sex types (Jacobs & Wade, 2003). Variation in sex ratios thus offers insights for our understanding of the evolution of nuclear-cytoplasmic gynodioecy (Frank & Barr, 2001; Jacobs & Wade, 2003). Species that exhibit sex ratio variation across ecological gradients provide particularly attractive model systems for empirical investigations of the evolution and maintenance of gender dimorphism (Geber *et al.*, 1999; Delph, 2003; Stehlik & Barrett, 2005).

Analysis of progeny sex ratios in several gynodioecious species has shown that the sex determination system is highly polymorphic involving complex nuclear-cytoplasmic gene interactions that challenge theoretical models (Charlesworth & Laporte, 1998; Jacobs & Wade, 2003; van Damme *et al.*, 2004). Cytoplasmic male-sterility (CMS) genes are mitochondrial mutants and, as such, predominantly maternally inherited (Couvet *et al.*, 1990; McCauley *et al.*, 2005). Several CMS types are generally found in natural plant populations (de Haan *et al.*, 1997c; Charlesworth & Laporte, 1998; Dudle *et al.*, 2001; Taylor *et al.*, 2001; van Damme *et al.*, 2004). Bi-parentally inherited nuclear genes act to restore male fertility when in combination with a particular CMS type (Hanson & Bentolila, 2004). Hermaphrodites typically carry a CMS gene in combination with appropriate restorers, as inferred from the commonly observed segregation of females in families of hermaphrodite seed-parents (Gouyon & Couvet, 1985; Charlesworth & Laporte, 1998; Dudle *et al.*, 2001; van Damme *et al.*, 2004), and multiple restorer loci appear to be involved in the restoration of a given CMS type. Restorer genes may act epistatically or independently, and several restorer alleles have been found to be dominant (Koelewijn & van Damme, 1995; de Haan *et al.*, 1997a; Charlesworth & Laporte, 1998; Bailey & McCauley, 2005). The presence of appropriate restorers may thus be estimated from progeny sex ratios (Manicacci *et al.*, 1997; Bailey, 2002; Koelewijn, 2003). These findings raise the question of how such CMS-restorer gene systems are distributed among natural populations and how this distribution is related to evolutionary processes in gynodioecious sex systems (Frank & Barr, 2001; Charlesworth, 2002; van Damme *et al.*, 2004; Olson *et al.*, 2005).

Negative pleiotropic fitness effects of nuclear restorers (costs of restoration) are an important component of theoretical models of stable cytonuclear gynodioecy (Bailey *et al.*, 2003; Jacobs & Wade, 2003) and may depend on cytoplasmic background, because costs of mismatched restorer alleles are no longer offset by the benefits of restoration (Charlesworth, 1981; Frank, 1989; Gouyon *et al.*, 1991; Jacobs & Wade, 2003). Selection should thus result in spatial covariation of CMS types and appropriate restorers. Consistent with these expectations,

the frequency of an otherwise rare restorer allele has been found to be increased in patches of the corresponding CMS type within a population of *Plantago lanceolata*, as inferred from progeny sex ratios (van Damme, 1986). Accordingly, at the interpopulational scale, one would expect higher restoration rates in within than in among population crosses, depending on the strength of selection and its balance with mutation, gene flow and stochastic loss of diversity.

Environmental conditions have been found to covary with population sex ratios in several gynodioecious species. Low hermaphrodite frequencies are often associated with apparently harsh or low quality habitats; e.g. with drier sites across moisture gradients (Darwin, 1877; Alonso & Herrera, 2001; Delph, 2003; Barr, 2004b; Vaughton & Ramsey, 2004) or with higher alpine sites across altitudinal gradients (Schrader, 1986; Delph, 1990; Alatalo & Molau, 1995; Puterbaugh *et al.*, 1997). Indeed, mountain ranges provide particularly suitable study areas with naturally replicated ecological gradients over short geographic distances (Körner, 1999). In the following, we outline hypotheses on how ecological variation across subalpine to alpine altitudinal gradients may cause hermaphrodite frequency to decrease with increasing elevation and on how offspring sex ratios may help with an understanding of the underlying evolutionary processes.

First, environmental factors that vary across altitudinal gradients, such as temperature or solar irradiation, might affect observed sex ratios via direct impacts on the expression of sex morphs. Nuclear-cytoplasmic sex determination, as it is the result of complex gene interactions, has a quantitative component (Ehlers *et al.*, 2005) that makes it susceptible to environmental impact. For instance, temperature has been shown to have a substantial influence on sex determination in partially male-sterile individuals of *P. coronopus* (Koelewijn & van Damme, 1996). The magnitude and directionality of effects of temperature on sex determination vary among gynodioecious species (Kaul, 1988).

Second, added stochasticity due to increased population isolation or turnover at high elevation sites could lead to decreased hermaphrodite frequencies owing to a lack of appropriate restorer alleles. Genetic drift in small and/or isolated populations is expected to lead to reduced diversity at restorer loci, which, in turn, results in increased female frequencies within adult populations or among their offspring (Byers *et al.*, 2005; Nilsson & Agren, 2006). Similarly, founder effects may cause a temporary absence of appropriate restorer alleles and, consequently, high proportions of females in young populations, as documented in early-successional populations of *T. vulgaris* (Gouyon & Couvet, 1985; Manicacci *et al.*, 1996). Stochastic processes might thus cause a sex ratio gradient in the case that their magnitude is correlated with environmental gradients (e.g. with latitude; Nilsson & Agren, 2006). If this is the case along an altitudinal gradient, one would expect a positive correlation between sex ratios of adult populations and sex ratios of their offspring (Couvet *et al.*, 1986; Byers *et al.*, 2005). Further, in among population crosses, one would predict a generally reduced restoration ability of hermaphrodites originating from high elevation sites, as compared to those from low elevation sites.

Third, varying ecological conditions may cause differential selection along altitudinal gradients, potentially affecting the relative seed fitness of the two sex types and, thereby, population sex ratios (Delph, 1990; Jacobs & Wade, 2003). If population sex ratios are stable, relative differences in the female fertility of the two sex types in natural populations may be inferred from adult and offspring sex ratios (Couvet *et al.*, 1986). A positive correlation between the relative female fertility of hermaphrodites and the proportion of hermaphrodites among adults would provide evidence of selective processes affecting sex ratio variation among populations. In this case, one would expect hermaphrodite seed-parents to contribute less to adult populations at higher than at lower altitudes.

We explored proximate causes of sex ratio variation among late-successional populations of the widespread gynodioecious thyme species *Thymus praecox* agg. across subalpine to alpine altitudinal gradients in the European Alps. We tested the stability of sex determination in a transplant experiment and, in a population genetic approach, estimated the diversity and spatial distribution of cytoplasmic and nuclear sex-determining genes and the relative seed fitness of sex types from sex ratio data. We analysed sex ratios of adult populations, of offspring from open pollination and from controlled within and among population crosses to address the following questions: (1) Do population sex ratios in *T. praecox* agg. vary across altitudinal gradients? (2) What factors determine the spatial variation of sex-determining genes among populations? (3) What are the relative roles of stochasticity and selection for sex ratio variation?

Materials and methods

Study species

Thymus praecox agg. Opiz ampl. Jalas (Lamiaceae) is widespread in the European Alps from subalpine to alpine altitudes and is found on rocky surfaces and in pastures (Jalas, 1970). It is a long-lived perennial forming carpets with numerous inflorescences and large floral displays, and with marginal, creeping sterile shoots (Jalas, 1970). Plants flower from June to August in populations of the Swiss Alps. The species is gynodioecious. Females have smaller flowers than hermaphrodites, and females show different degrees in the reduction of male organs; some females still bear white sterile anthers while hermaphrodites harbour purple fertile anthers (U. Landergott, pers. observ.). Hermaphrodites are self-compatible, but hermaphrodite flowers are highly protandrous and their styles are long protruding at the time the stigmatic surface becomes receptive. In contrast, the stigmas of females are already receptive when flowers open (U. Landergott, pers. observ.). Thyme produces four ovules per flower.

Thymus praecox agg. is a tetraploid with tetrasomic inheritance (Landergott *et al.*, 2006). High morphological, biochemical and genetic diversity all point to a reticulate evolutionary history of this aggregate species (Jalas & Kaleva, 1970; Bischof-Deichnik *et al.*, 2000; Landergott *et al.*, 2006). Our study populations are best assigned to *T. praecox* Opiz ssp. *polytrichus* (A. Kerner ex Borbás) Jalas (Tutin *et al.*, 1972). However, as the subspecific

boundaries are vague (Jalas, 1970) and given the high morphological diversity present within populations (U. Landergott, unpubl. data), we only refer to *T. praecox* agg. in this study.

Natural populations: altitudinal gradients

We chose study areas replicated in different regions of the Swiss Alps according to the following two criteria: (1) *Thymus praecox* agg. had to be abundant and widely distributed within study regions across a subalpine to alpine altitudinal gradient; (2) study populations within regions had to be spatially non-isolated and located in late-successional habitats (pasture, rock steppe or alpine grassland) at sites characterised by similar slopes and exposition (mainly south). These criteria were met in the five study regions Flimserstein [F], Langwies [L], Piora valley [P], Säntis [S] and Zwinglipass [Z] (Appendix S1). The study regions S and Z were both located in the Northern Calcareous Alps separated from each other by 4 km beeline, all other regions were geographically remote from one another with the largest distance of 90 km between S/Z and P in the Lepontine Alps. In the five study regions, we estimated population sex ratios and ecological variables at a total of 30 study sites during peak flowering in the years 2000 to 2002. At each site and on an area of 300 to 400 m^2, we recorded the frequency of sexual phenotypes (females, hermaphrodites and intermediates) along nine to 17 randomly placed 5 m-transects, so that on average 85 individuals were sampled per site (Appendix S1). For each transect (strip of a 5 m-measuring tape), the proportion covered with thyme was recorded in order to estimate population density. For each population, we quantified the heterogeneity in the distribution of sex types by calculating the average absolute deviation of transect sex ratios from the mean population sex ratio (intermediates were designated hermaphrodite to calculate sex ratios). Furthermore, we classified (five ranks) the cover of the vegetation adjacent to each of the recorded individuals of *T. praecox* agg. in order to estimate the mean vegetation cover in the vicinity of thyme plants at each study site. Finally, we recorded plant species co-occurring with thyme along the transects (on average 30 species per site) in order to calculate site means of Landolt's ecological indicator values (equivalent to Ellenberg indicator values; Diekmann, 2003). These values indicate plant preferences for levels of humidity [f], soil reaction [r], nutrients [n], soil humus content [h], soil dispersion [d], light [l], temperature [t] and continentality [k] (Landolt, 1977).

We abandoned analysis of a sixth region, Goms, because *T. praecox* agg. turned out to be patchily distributed at higher altitudes and because we observed hybridisation with *T. oenipontanus* Heinr. Braun in the vicinity of lowermost study sites (Moser *et al.*, 2003). Nevertheless, we report in Appendix S1 our findings from late-successional study sites in the Goms region as we could compare them to an early-successional site [GP]. This pioneer population of *T. praecox* agg. grew in a man-made flood protection area and had a maximal age of 14 years.

Low vs. high altitudinal populations

In each of the five study regions F, L, P, S and Z we chose both a low [L] and high [H] altitudinal population of *T. praecox* agg. for further comparative investigations (abbreviation ZH thus refers to the high altitudinal study population from Zwinglipass; Appendix S1). Within each of these ten natural populations, 15-20 individuals displaying a minimum of five inflorescences were randomly chosen as focal plants, as well as the nearest individual of the complementary sexual phenotype each. A total of 350 focal plants were permanently marked. In the years 2001 to 2003, mostly one year after the sex ratio of a given population had been estimated from transects, the neighbourhood sex ratio of each focal plant was determined by recording the sexual phenotypes of the six nearest neighbours. Population sex ratios in the neighbourhoods of females and hermaphrodites were calculated separately to estimate nonrandom distribution of sex types. The average of both female and hermaphrodite neighbourhoods provided another overall estimate of adult sex ratios.

In addition, open pollinated fruits were collected from the permanently marked focal plants. In August 2003 (populations LH, LL, PH, PL, SL and ZL) and in April 2004 (SH and ZH), up to 66 seeds per mother were sown in a greenhouse at the Botanical Garden of Zurich in plastic multi-pot plates (up to 33 seeds per 4.5 cm-diameter pot containing a standard potting mixture). Germination rates were recorded (U. Landergott, unpubl. data) and seedlings were thinned to about ten per pot. For an average of twelve seed-parents per sex type per population from each of the four regions L, P, S and Z, up to five half-sibs each were pricked out and individually grown outdoors in 6 cm-diameter biodegradable pots containing a mixture of topsoil and sand (2:1). In October 2003 and in July 2004 (populations SH and ZH), a total of 923 offspring were bedded out to the experimental field at the alpine garden of Arosa (see below). 97.3% of these offspring survived till summer 2005. By then, a total of 845 offspring, on average 53 per maternal sex type per population, were sexed. Intermediates were designated hermaphrodite to calculate offspring sex ratios.

Low and high altitudinal experimental fields

We set up a transplant experiment using clonally propagated genotypes to determine the stability of sexual phenotypes under different environmental conditions and across study years. We cut creeping shoots of a total of 135 permanently marked individuals during the flowering season 2001 from populations PL, PH, ZL and ZH, and in 2002 from populations SL and SH, respectively. Additionally, shoots were also collected in low altitudinal populations from four individuals of intermediate sexual phenotype or with extreme flower sizes, because such plants are more likely to show labile sex expression (Ehlers *et al.*, 2005). Cuttings were further divided into two ramets each and propagated in 15 cm-diameter clay pots containing a mixture of 50% topsoil and 50% sand at the Botanical Garden of Zurich. In October 2001 (2002 for populations SL and SH), a total of 139 genotypes (278 ramets) were bedded out each to a low and high altitudinal experimental field near the Botanical Garden of Zurich (Burghölzli, 460 m a.s.l., Swiss coordinates: 685150.245300) and Arosa (Maran, 1850 m a.s.l., 771670.184880),

respectively. Ramets were completely randomised within experimental fields and spaced from each other by a distance of 50-60 cm. The plants mainly rooted within pots till the first flowering season, but later extended growth into the soil of the experimental fields. At the low altitudinal experimental field, some dense cushions suffered from fungal infection from the second flowering season onwards. The flowering status and the sexual phenotypes of the ramets were recorded in the years 2002 to 2004.

Crossing experiment

We investigated the spatial structure of sex-determining genes in *T. praecox* agg. in a controlled crossing experiment on the four populations PL, PH, ZL and ZH. The two regions P and Z were geographically remote. Within regions, the low and high altitudinal study populations had distinctly different sex ratios (Appendix S1) and were separated from one another by 1.5-3 km. The five pollination treatments were as follows: on hermaphrodites, self-pollination (HS) and within population outcrosses (HO) and, on females, within population crosses (FP), crosses among altitude within region (FA) and crosses among region within altitude (FR). The objective was to use each parental individual once in each treatment in order to be able to estimate effects of dams and sires, respectively. A total of 32 females, eight from each population, were used as maternal plants in treatments FP, FA and FR. Similarly, we intended to use 32 hermaphrodites both as maternal plants in treatments HS and HO and as pollen donors once in all of the five treatments. A fully balanced crossing scheme was achieved for the three F-treatments, comprising a total of 96 families. The two H-treatments yielded additional 64 families. Here we focus on the analysis of progeny sex ratios from treatments FP, FA and FR, but progenies from treatments HS and HO were included to analyse potential environmental effects on sex expression.

The controlled crossing experiment was performed in 2003 (low altitudinal field: May to June; high altitudinal field: June to July). On maternal individuals, groups of six to ten inflorescences were cut free prior to anthesis and marked; one group per treatment and a control. Pollinators were excluded by cages (30 × 30 × 15 cm) made from a wire netting frame and covered with fly screen. Cages reduced light levels to some extent, but did not affect flowering as compared to uncaged inflorescences. Per full-sib family, hand pollinations were carried out every two to three days, during the entire flowering season in order to ensure sufficient seed production. Pollen was collected on small pieces of nylon thread and transferred to receptive stigmas. Fruits were collected from June to July at the low and from July to August at the high altitudinal field. Mean seed set per fruit of females was not affected by the different crossing treatments (U. Landergott, unpubl. data).

Seeds were sorted according to their number per fruit and standardised mixtures of seed classes were used for sowings. Where sufficient seeds were available, half of the seeds per crossing family were subjected to a cold treatment during four weeks at 4°C and additional ten days at –15°C prior to the sowing in early April 2004. Two pots per crossing family were thus germinated (for details see above). Germination success did not differ between the pollination

treatments FP, FA and FR (U. Landergott, unpubl. data). Up to 15 offspring were pricked out per crossing family. In mid-June 2004, 1291 offspring, half with and half without seed cold treatment, were bedded out to the low altitudinal experimental field, separated from one another by a distance of 25 cm. Another 1058 offspring were bedded out by end-July 2004 to the high altitudinal experimental field, separated by 20 cm. Few individuals that died or were destroyed by wild animals were replaced if possible (surrogates were included in sex ratio analysis, but excluded for calculation of the proportion of non-flowering plants). The survival rate was similar among cross treatments on females (FP: 0.95; FA: 0.93; FR: 0.96). In summer 2005, flowering offspring were sexed. Intermediate sexual phenotypes with predominantly hermaphrodite flowers were designated hermaphrodite; those with predominantly female flowers were designated female in the calculation of progeny sex ratios.

Molecular genetic analyses of a subsample of ten full-sib families confirmed the accuracy of the artificial crosses (Landergott *et al.*, 2006). However, in a few cases, we suspected mislabelling of individuals or contaminants among crosses. Therefore, we checked the parentage of additional 85 offspring by microsatellite-genotyping. Four contaminants were consequently excluded and 25 individuals were re-assigned for sex ratio analyses (mainly from three families: outcross treatments on two hermaphrodites for which pollinators were observed within the cages and one cross on a female attributable to an erroneous hand-pollination event).

Statistical analysis

Sex ratio data are best expressed as proportions (Wilson & Hardy, 2002). Such grouped binary data are preferably analysed by means of generalised linear modelling with a binomial error term and a logit link function that constrains the predicted values to lie within realistic bounds (Wilson & Hardy, 2002). We used the generalised liner model (GLM) procedure implemented in the statistical package JMP (version 6.0; SAS Institute Inc., Cary, NC, USA) for analysis of proportion data. Over- or underdispersion was estimated by dividing the Pearson's χ^2-value by the residual degrees of freedom and the likelihood ratio χ^2-statistics for effect tests were adjusted by rescaling. When random factors were involved, we constructed *F*-tests for affected fixed effects using the likelihood ratio χ^2-statistics analogous to ANOVA sums of squares, with the appropriate term for the denominator specified according to the ANOVA method (Taylor *et al.*, 2001; Quinn & Keough, 2002). Models were checked by inspection of goodness-of-fit, by the magnitude of overdispersion and by residual analysis (Quinn & Keough, 2002; Wilson & Hardy, 2002).

We performed a principal component analysis (PCA) to identify independent ecological gradients across 30 natural populations of *T. praecox* agg. from five study regions (Legendre & Legendre, 1998). Principal components were extracted from the correlation matrix of twelve ecological descriptors (altitude, population density and heterogeneity, vegetation cover and eight ecological indicator values), and the factor scores for each study site were recorded using SPSS (version 11.0.4 for Macintosh; SPSS Inc., Chicago, IL, USA). Axes with eigenvalues larger than 1.0 were used for further analyses. A GLM with the factor scores of the principal

components as continuous predictors and region as a categorical predictor was performed to test for effects of ecological gradients on the proportion of hermaphrodites in natural populations. Preliminary models revealed no significant principal component by region interactions ($P > 0.35$ in all cases), and these interaction terms were thus omitted from analysis.

Neighbourhood and offspring sex ratios from natural populations were analysed by a series of structurally analogous, partly nested GLMs. To investigate the effect of sampling method (transect vs. neighbourhood) on estimates of adult sex ratios, the fixed factor altitude was tested over the random effect of population nested within altitude (F-test); the sampling method and its interaction with altitude were fixed effects. A second analysis comprised focal plant gender as a fixed effect to test for nonrandom spatial distribution of sex types and its interaction with altitude. Similarly, we tested for effects of altitude and maternal sex on sex ratios of offspring from open-pollination. Finally, we tested for a difference between the sex ratios of adult plants in a population and those in the offspring of females in order to examine whether hermaphrodites had a significant female function.

We estimated the relative female fertility of hermaphrodites in the different populations from sex ratio data using a descriptive model that accounts for the frequency of the sex types among the adults (Couvet *et al.*, 1986):

$$A' = (RFF_H \times A \times H + (1 - A) \times F)/(RFF_H \times A + 1 - A)$$

where A = the observed sex ratio of the adult population; F = the sex ratio in the offspring of females; H = the sex ratio in the offspring of hermaphrodites; RFF_H = the relative female fertility of hermaphrodites (seed production and germination success) as compared to females; A' = the expected adult sex ratio in the next generation, provided that there are no differences in the survival rate among the offspring types. Under stable population sex ratios ($A' = A$) and assuming equal chances of reproduction among germinated offspring (scenario A), the expected RFF_H may thus be derived from the above formula. However, in the context of inbreeding and/or cost of restoration that will affect offspring of hermaphrodite seed-parents more severely than offspring of females (Jacobs & Wade, 2003; Bailey & McCauley, 2005), it may be more sensible to assume that the offspring of female seed-parents will primarily contribute to the next generation and that hermaphrodite seed-parents will mainly contribute hermaphrodite offspring ($H = 1$ and $A' = A$; scenario B). We calculated the expected RFF_H under both scenarios and performed one-tailed paired t-tests to check whether RFF_H was lower at higher altitudes. For population PL, the average sex ratio of the controlled crosses HS and HO (proportion of hermaphrodites = 0.88) was used as H in scenario A because this estimate appeared more reliable than the one from the natural population (small sample size; see below).

Analyses of progeny sex ratios from controlled crosses on females aimed at testing for patterns of variation in sex-determining genes at different spatial scales. We assumed effects of dams to mainly reflect variation among CMS types and effects of sires to reflect variation among nuclear restorers (Taylor *et al.*, 2001). Therefore, separate models were constructed including the original altitude and the region of origin of either the dams or the sires. Preliminary, full-factorial GLMs were fitted with the three factors pollination treatment,

original altitude and region in order to check for significant interactions of original altitude and region with pollination treatment. As no such interactions were detected ($P > 0.35$ in all cases), they were excluded, and two models were fitted including the dams and sires as blocking factors, respectively. A sequence of planned contrasts of pollination treatments was performed based on the model that accounted for the effects of individual dams. First, the two among population crossing treatments (FA vs. FR) were compared to test whether the restoration ability of sires depended on the geographic distance between populations. Depending on the outcome of this test, we consequently contrasted the within population crosses (FP) with either the FA-crosses or the pooled among population crosses (FA and FR) to test for enhanced restoration ability of sires within populations. These analyses were complemented by the calculation of pairwise Pearson correlations between sex ratios of maternal half-sib families among the three crossing treatments. Analogous analyses of arcsine-transformed data by means of general linear models with a normal error distribution gave very similar results (not shown) as the above generalised linear model analyses of the controlled crosses. Therefore, a general linear model on arcsine-transformed data was performed to estimate the variance components (REML method) attributable to dams, sires and their interaction (residual). Finally, because observed progeny sex ratios may have been biased in case where sexes differed in the probability of flowering (being more likely to affect hermaphrodites than females; Jacobs & Wade, 2003), we re-ran the analyses of controlled crosses on a manipulated data set in which non-flowering individuals were designated hermaphrodite.

Logistic regression on binary data was used to test for environmental effects on sex determination (hermaphrodite or female) among progenies from all five pollination treatments. The effect of seed cold treatment on sex expression among progenies reared in the low altitudinal experimental field was tested in a model including pollination treatment (fixed factor) and crossing family nested within pollination treatment (random; REML estimation). An analogous model was used to test for an effect of experimental altitude on sex expression.

Results

Adult sex ratios in natural populations

Intermediate sexual phenotypes, i.e. individuals harbouring flowers with both sterile and fertile anthers, or inflorescences with both female and hermaphrodite flowers, were rarely observed in natural populations of *T. praecox* agg. (proportion of intermediates: mean = 0.005, maximum = 0.047; Appendix S1). Population sex ratios (proportion of hermaphrodites) of 30 late-successional populations of *T. praecox* agg. from five regions in the Swiss Alps ranged from 0.73 to 0.40 (Appendix S1). Four main ecological gradients (principal components) were extracted from the set of twelve ecological variables characterising the study sites (Table 1). PC1 represented the altitudinal gradient, with the average temperature during the vegetation period decreasing and with the humidity increasing with increasing altitude. PC2 corresponded to a gradient in resources, with less light being available at nutrient richer sites. PC3 reflected

soil conditions indicating that *T. praecox* agg. grew in relatively denser vegetation in calcareous areas as compared to siliceous areas. PC4 represented the population structure of *T. praecox* agg. indicating that the distribution of the two sex types was more heterogeneous in populations with lower plant density (Table 1). One out of the four ecological variables, the altitudinal gradient, was a highly significant predictor of population sex ratio (Table 2); the proportion of hermaphrodites decreased with increasing altitude (Fig. 1). None of the remaining three ecological gradients influenced population sex ratios significantly, and there was no significant variation among the five replicate study regions (Table 2).

There was no significant difference in estimates of adult sex ratios between methods (transects vs. neighbourhoods) and, thus, among study years (sampling method: $\chi^2_1 = 0.58$, $P = 0.446$). This GLM analysis confirmed the significant effect of altitude ($F_{1,8} = 39.72$, $P = 0.0002$), but the difference between altitudes was less pronounced in the neighbourhoods' data set than in the transect data set (interaction altitude × sampling method: $\chi^2_1 = 4.40$, $P = 0.036$; Table 3; Appendix S1). Variation in neighbourhood sex ratios indicated that the sex types tended to be nonrandomly distributed within populations (focal plant sex: $\chi^2_1 = 33.06$, $P < 0.0001$). The patchiness of sex types was similar at low and high altitudes (interaction altitude × focal plant sex: $\chi^2_1 = 0.17$, $P = 0.680$).

The early-successional, low altitudinal population GP exhibited a relatively low proportion of hermaphrodites (0.35), as compared to the high proportions of hermaphrodites found at low altitudinal, late-successional populations of *T. praecox* agg. in the same region (Appendix S1). This finding suggested that the population sex ratio in *T. praecox* agg. is affected by founder effects in a similar way as in *T. vulgaris*.

Stability of sex morphs

Ramets of a total of 133 randomly sampled genotypes from six natural populations of *T. praecox* agg. flowered from early May to late June at the low altitudinal and from mid-June to early August at the high altitudinal experimental field. Seven of these genotypes could only be sexed in one of the two fields. The sexual phenotype was stable under differing environmental conditions and among study years in all but two genotypes. One hermaphrodite from population SL and one female from population ZL showed reversible transitions to intermediate sexual phenotypes (Appendix S2). Four additionally transplanted genotypes of presumed intermediate state showed labile sex as well, but no trend in the change of sex was evident across experimental altitudes or study years (Appendix S2).

Sex determination among progenies from controlled crosses was neither affected by cold temperature treatment of seeds (logistic regression: likelihood ratio $\chi^2_1 = 0.41$, $P = 0.522$, $n = 937$ observations) nor by the environmental conditions at the two experimental fields (logistic regression: likelihood ratio $\chi^2_1 = 0.47$, $P = 0.494$, $n = 1895$ observations).

Sex ratio variation among offspring from natural pollination

Almost all (96%) of the offspring from open-pollination in populations LH, LL, PH, PL, SL and ZL, and 84% of the younger offspring from populations SH and ZH flowered in summer 2005. Intermediate sexual phenotypes were rarely observed among offspring from natural populations of *T. praecox* agg. (proportion of intermediates: 0.013, $n = 845$). Hermaphrodites were more frequent than females among offspring of hermaphrodite mothers and, likewise, females were more frequent than hermaphrodites among offspring of females (maternal sex: $\chi^2_1 = 47.93$, $P < 0.0001$; Table 3). However, there was no significant effect of altitude on offspring sex ratio ($F_{1,6} = 0.21$, $P = 0.661$), and there was also no significant interaction between altitude and maternal sex ($\chi^2_1 = 0.83$, $P = 0.363$). The proportion of hermaphrodites among offspring of hermaphrodite seed-parents was consistently higher than in adult populations, except for population PL, for which the estimate of offspring sex ratio was based on a relatively small sample size (seed-parents: $n = 10$; flowering offspring: $n = 42$). Similarly, the proportion of hermaphrodites in the offspring of females was lower than that in adult populations in all but one high altitudinal population (SH; generation: $\chi^2_1 = 44.29$, $P < 0.0001$; altitude × generation: $\chi^2_1 = 5.49$, $P = 0.019$; Table 3). The average relative female fertility of hermaphrodites inferred from sex ratio data was 0.70 under scenario A (assuming stable population sex ratios and equal survival of all offspring) and 0.25 under scenario B (stable sex ratios, superior seed fitness of females and hermaphrodite seed-parents contributing only hermaphrodites to the next generation). Under both scenarios, the expected relative female fertility of hermaphrodites was consistently lower in high altitude populations than in low altitude populations from regions L, P and S, but not in region Z (one-tailed *t*-tests for an effect of altitude; scenario A: $P = 0.134$; scenario B: $P = 0.094$; Table 3).

Sex ratio variation among progenies from controlled crosses

A total of 1219 progeny from 96 controlled crosses on females flowered, 89% of the progenies from the within population crosses (treatment FP), 95% of those from the among altitude within region crosses (FA) and 92% of those from the among region within altitude crosses (FR). The frequency of intermediate sexual phenotypes was low and in the order of magnitude of that found in the offspring from natural populations, but the mean proportion of intermediates slightly increased with increasing geographic distance between cross parents (Fig. 2a).

Three out of the 32 female seed-parents (dams), one each from population PH, ZL and ZH, did not segregate any hermaphrodite in all of the three crossing treatments (Fig. 3). Out of the 32 hermaphrodite pollen-parents (sires), two, one each from population PH and ZH, entirely failed to restore the male function in all of the crosses with three different females each (Appendix S3). Overall, progeny sex ratios were female biased and within population crosses gave similar proportions of hermaphrodites as those found among offspring of naturally pollinated females (Fig. 2b; Table 3). Dams accounted for 22% of the variance in sex ratios

among the 96 crosses, sires for 4% and the residual 74% was attributable to sire × dam interactions (variance component ± SE: 0.029 ± 0.019 for dams, 0.005 ± 0.014 for sires, 0.097 ± 0.019 for residual).

The geographic source of dams had no significant effect on progeny sex ratios (dam altitude: $\chi^2_1 = 0.01$, $P = 0.915$; dam region: $\chi^2_1 = 0.50$, $P = 0.477$; dam altitude × region: $\chi^2_1 = 0.82$, $P = 0.364$; in a GLM accounting for effects of pollination treatment and of individual sires). Similarly, the altitude from which the sires were sampled had no significant effect on their restoration ability (Table 4). The effects of sire region and sire population (interaction altitude by region) were not statistically significant either (Table 4). However, removing these latter effects from the analysis increased both the deviance and the overdispersion of the GLM, potentially indicating a biologically meaningful structure in the data set.

The pollination treatments showed a marginally significant overall effect on progeny sex ratios (Table 4). In among population crosses, the geographic distance between cross parents (within vs. among regions) did not significantly affect progeny sex ratios (Fig. 2b; Table 4). However, within population crosses produced significantly higher proportions of hermaphrodites than the pooled among population crosses (Fig. 2b, 4; Table 4). In agreement with these results, the pairwise correlations between sex ratios of maternal half-sib families were not significant for treatments FP and FA ($r = -0.01$, $P = 0.936$) and only marginally significant for treatments FP and FR ($r = 0.34$, $P = 0.057$), while there was a positive correlation for the among population cross treatments FA and FR ($r = 0.39$, $P = 0.028$; Fig. 3).

Designating all non-flowering plants hermaphrodite led to qualitatively similar, but more pronounced results (GLM analogous to that presented in Table 4; sire altitude: $\chi^2_1 = 0.07$, $P = 0.797$; sire region: $\chi^2_1 = 3.36$, $P = 0.067$; sire altitude × region: $\chi^2_1 = 2.13$, $P = 0.144$; pollination treatment: $\chi^2_2 = 8.07$, $P = 0.017$). A potential female bias in sex ratio estimates due to non-flowering individuals would thus not change our main results.

Discussion

The results of our study raise an immediate question: why are there more females at higher altitudes in gynodioecious *T. praecox* agg.? The correlation between sex ratios and altitude, replicated in geographically remote valleys, precludes that sex ratio variation among late-successional populations of *T. praecox* agg. is attributable to differing phases of limit cycles in a dynamic equilibrium of sex-determining genes (Gouyon *et al.*, 1991; de Haan *et al.*, 1997b). Furthermore, low proportions of hermaphrodites did not generally result from added stochastic disturbance of the equilibrium between sex-determining genes at higher altitudes, since there was no evidence of reduced compatibility or diversity of sex-determining genes in most of the high altitudinal populations. Additionally, the observed sex ratio gradient did not result from impacts of environmental conditions on sex determination, as the expression of sex morphs was stable and independent of temperature and altitude in *T. praecox* agg. Together these

findings suggest that our study system met conditions favourable for examining effects of selection on the gynodioecious cytonuclear polymorphism.

Characteristics of nuclear-cytoplasmic inheritance of sex

We did not attempt to explicitly test for nuclear-cytoplasmic inheritance of sex, which would require conducting reciprocal crosses. However, sex ratio variation in *T. praecox* agg. showed several features that typically result from nuclear-cytoplasmic inheritance. First, offspring tended to be of the same sex as the seed-parents (Table 3; Charlesworth & Laporte, 1998). Second, different CMS types and corresponding subsets of nuclear restorer alleles existed in natural populations, as inferred from varying restorability of individual females or restoration ability of individual hermaphrodites in different crosses, respectively (Fig. 3; Appendix S3; Manicacci *et al.*, 1997; Gigord *et al.*, 1998; Dudle *et al.*, 2001). Third, a considerable proportion of the total variance being attributable to dam effects suggested that CMS types differed in their likelihood of restoration, and an even higher proportion of the variance being attributable to dam × sire interactions illustrated cytonuclear epistasis (Fig. 3; Gigord *et al.*, 1998; Taylor *et al.*, 2001). Finally, the two sex types tended to be nonrandomly distributed within populations, which calls attention to appropriate spatial sampling scales and frequency dependent processes within populations (van Damme, 1986; Graff, 1999; Frank & Barr, 2001; Olson *et al.*, 2005).

Diversity of sex-determining genes and stochastic evolutionary processes

Offspring sex ratios reflect the frequencies of alleles of the sex determining genes in cytonuclear gynodioecious species (Couvet *et al.*, 1986; Byers *et al.*, 2005). Two lines of evidence conflict with the hypothesis that variation in genetic diversity at sex-determining loci was responsible for the observed sex ratio variation among populations of *T. praecox* agg. across altitudinal gradients. First, offspring sex ratios of natural populations were consistent among altitudes and, thus, independent of adult sex ratios. This indicates that nuclear restorers matched CMS types equally well within low and high altitudinal populations, precluding that added founder effects at higher altitudes caused the observed sex ratio gradient. Second, restoration ability in controlled within and among population crosses was not significantly different between sires from low and high altitudinal populations, indicating that the diversity at nuclear restorer loci was not generally reduced at higher altitudes. We conclude that sex ratio variation among late-successional populations of *T. praecox* agg. across subalpine to alpine altitudinal gradients was not generally governed by stochastic evolutionary processes.

However, results from one study region, Z, suggested that reduced diversity at sex-determining loci might have contributed to sex ratio variation in this region. Offspring sex ratios of both females and hermaphrodites consistently covaried with adult population sex ratios in region Z (Table 3; Fig. 4). Sires from the high altitudinal population ZH showed lower restoration ability than those from the low altitudinal population ZL (treatments FP and FA in populations ZL and ZH; Fig. 3), and population ZH exhibited the lowest overall restoration

ability (Fig. 4). Indeed, calculations of expected relative female fertility of hermaphrodites in populations ZL and ZH indicated that the differences in segregation ratios could explain the difference in adult sex ratios between these two populations (Table 3).

Spatial correlations between CMS types and nuclear restorers, local adaptation and cost of restoration

Progeny sex ratios of crosses that represent different geographic distances provide insights into the spatial distribution of nuclear and cytoplasmic sex determining genes, which sheds light on their coevolution (Taylor *et al.*, 2001; Bailey & McCauley, 2005). Spatial correlations between sex-determining genes may result from two inherently different evolutionary scenarios (Charlesworth, 2002). (1) Frequent and fairly rapid evolutionary turnover of CMS types may lead to genetic differentiation in the cytonuclear system among reproductively isolated populations (Frank, 1989; Barr, 2004a). If different populations comprise different CMS types and their matching restorer alleles, then crosses among populations should yield female biased progeny because of a mismatch between CMS and restorer genes. High female frequencies are common in agricultural hybrid crosses (Kaul, 1988) and have also been reported in hybrids between different flower colour morphs in the gynodioecious *Nemophila menziesii* (Barr, 2004a). (2) Particular CMS types and their restorers may be selectively maintained in gynodioecious species for long time periods (Städler & Delph, 2002). Spatial variation in the frequency of CMS types, in combination with costs of restoration affecting mismatched restorer alleles, should lead to locally adjusted restorer frequencies (Jacobs & Wade, 2003) and, thus, female biased progeny in among population crosses.

Sex ratio data seemed to reject scenario (1) in *T. praecox* agg: There was no evidence of spatial correlations between sex-determining genes at the regional scale; crosses among geographically remote regions did not yield lower hermaphrodite frequencies than within region crosses (Fig. 2b; Table 4). Moreover, the geographic source of CMS types, as represented by dams, did not explain variation in progeny sex ratios. In fact, in all of the three crossing treatments, certain crosses (CMS-restorer combinations) showed a high proportion of hermaphrodite progeny (Fig. 3), indicating that both CMS and nuclear restorer alleles were geographically widespread in *T. praecox* agg. These findings suggested long-term maintenance of nuclear and cytoplasmic factors controlling the sexual polymorphism in tetraploid *T. praecox* agg., hence corroborating recent molecular genetic results in *S. acaulis* (Städler & Delph, 2002; Klaas & Olson, 2006).

At a local spatial scale, however, a positive correlation between cytonuclear sex-determining genes was evident in *T. praecox* agg, as expected under scenario (2): within population crosses yielded higher proportions of hermaphrodites than among population crosses (Fig. 2b; Table 4). In other words, the restoration ability of particular subsets of restorers, as represented by sires, was highest in within population crosses, consistently so even in the genetically depauperate population ZH (Fig. 4). These findings are in line with theoretical predictions of local adaptation between antagonistically interacting systems

(Gandon *et al.*, 1996). Indeed, cytonuclear gynodioecy has often been seen as an example of a genomic conflict between cytoplasmic and nuclear genes, and parallels have been drawn with host-pathogen systems (Gouyon & Couvet, 1985; Couvet *et al.*, 1990; Frank & Barr, 2001). However, if there are deleterious fitness effects of nuclear restorers (costs of restoration), the cytonuclear coevolution is not consistent with simple antagonistic interactions (Jacobs & Wade, 2003). Our finding of locally adjusted frequencies of restorer alleles, or subsets of restorers, could indicate selection against mismatched restorers due to costs of restoration.

Comparable investigations on gynodioecious *S. vulgaris* (Bailey & McCauley, 2005) and *T. vulgaris* (Gigord *et al.*, 1998) did not detect spatial correlations between cytonuclear sex-determining genes, possibly because of the prevalence of stochastic evolutionary processes in the study populations. The contrasting results in *T. praecox* agg. have two implications for the understanding of the evolution of the nuclear-cytoplasmic sex system in our study species: that the spatial distribution of the sex-determining genes is affected by selective processes, and, in turn, that the evolutionary history of late-successional populations of this long-lived species allows for detecting the signature of selective forces in this gynodioecious system.

Sex ratios and functional gender

Estimating the functional gender of sex types from offspring sex ratios requires largely stable sex ratios of adult populations, as appears to be the case for the study populations of *T. praecox* agg. (except in region Z). The hermaphrodite frequency in the offspring of open-pollinated females was lower than the frequency of hermaphrodites in adult populations of *T. praecox* agg., suggesting that hermaphrodites will contribute to the next generation via their female reproductive function. Note that the absolute values of the expected relative female fertility of hermaphrodites in *T. praecox* agg. inferred from sex ratio data should be interpreted with caution, because the estimates depended on restrictive assumptions concerning survival and because they were derived from relatively small sample sizes. Nevertheless, the comparison of the two scenarios A and B (Table 3) illustrates the importance of understanding the survival rate of offspring in relation to their sex type and their maternal sex in natural populations (van Damme & van Damme, 1986). Moreover, diminished estimates of relative female fertility of hermaphrodites at higher altitudes implied that hermaphrodite seed-parents contribute less to adult populations at high altitude than at low altitude in three of the four study regions. The functional gender of hermaphrodites was thus more skewed towards maleness at higher than at lower altitudes due to differences in relative seed fitness (in addition to skeweness caused by population sex ratio per se; Delph, 2003). The relationship between altitude, population sex ratio and inferred relative seed fitness of hermaphrodites suggests that environmentally dependent selection affected sex ratio variation in *T. praecox* agg. along ecological gradients. Our findings thus add to an increasing number of studies supporting the hypothesis that ecological factors can influence selective forces acting on the nuclear-cytoplasmic sex system and, consequently, cause sex ratio variation in species with cytonuclear gynodioecy (Delph & Carroll, 2001; Delph, 2003; Barr, 2004b).

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Table 1 Factor loadings from PCA on twelve ecological descriptors recorded in 30 late-successional populations of *Thymus praecox* agg. from the Swiss Alps. PC1 explained 30.85% of the total variance (eigenvalue 3.70), PC2 28.87% (3.46), PC3 13.17% (1.58) and PC4 9.84% (1.18).

Ecological variable	PC1	PC2	PC3	PC4
Temperature (Landolt's t)	-0.96	0.16	0.10	0.12
Altitude	0.95	-0.19	0.08	-0.08
Humidity (Landolt's f)	0.83	0.49	0.13	-0.11
Nutrients (Landolt's n)	-0.14	0.89	-0.02	-0.07
Continentality (Landolt's c)	-0.36	-0.83	-0.26	0.06
Light (Landolt's l)	0.60	-0.72	-0.21	0.03
Reactivity (Landolt's r)	-0.15	0.13	-0.80	0.10
Vegetation cover	-0.20	0.22	0.75	0.08
Soil humus (Landolt's h)	0.49	0.32	0.69	0.09
Soil dispersion (Landolt's d)	-0.32	0.57	0.62	0.02
Population heterogeneity	-0.03	0.21	-0.04	-0.83
Population density	-0.21	0.10	-0.03	0.82

Table 2 Relationship between four independent ecological variables (principal components) and sex ratios of 30 natural populations of *Thymus praecox* agg. from the Swiss Alps. The five study regions were included as a blocking factor in the GLM analysis.

	Parameter estimate		Effect test		
Source	*ß*	SE	d.f.	χ^2	*P*
PC1 'altitudinal gradient'	-0.28	0.04	1	44.20	0.000
PC2 'resource availability'	0.07	0.05	1	2.02	0.155
PC3 'soil conditions'	-0.01	0.07	1	0.01	0.914
PC4 'population structure'	0.06	0.06	1	1.09	0.297
Region			4	1.80	0.773

Table 3 Adult and offspring sex ratios of open-pollinated females and hermaphrodites from low and high altitudinal natural populations of *Thymus praecox* agg. and inferred relative female fertility of hermaphrodites (RFF_H). Adult sex ratios were derived from neighbourhood sex ratios of focal plants (including seed-parents). Offspring sex ratios were estimated from a total of 42-62 offspring per sex type and population, of ten to13 seed-parents each. Expected RFF_H was calculated assuming stable adult sex ratios and equal survival of all offspring (scenario A) and superior survival of offspring from females and hermaphrodite seed-parents contributing only hermaphrodites to the next generation (scenario B).

	Proportion of hermaphrodites				
Population		Offspring from seeds of		Expected RFF_H	
abbreviation	Adults	Females	Hermaphrodites	Scenario A	Scenario B
FL	0.71	–	–	–	–
FH	0.54	–	–	–	–
LL	0.63	0.47	0.67	2.01	0.24
LH	0.57	0.47	0.79	0.36	0.19
PL	0.62	0.34	0.52	0.66	0.45
PH	0.46	0.36	0.87	0.29	0.22
SL	0.61	0.44	0.89	0.41	0.29
SH	0.52	0.52	0.78	0.00	0.00
ZL	0.67	0.47	0.79	0.81	0.30
ZH	0.48	0.31	0.65	1.06	0.35

Table 4 Generalised linear model (GLM) analysis on progeny sex ratios of 96 controlled crosses in *Thymus praecox* agg. from the Swiss Alps, testing for effects of the geographic source of hermaphrodite sires (altitude and region) and of the geographic distance between parents (pollination treatment; FP: within population; FA: among altitude within region; FR: among region within altitude) on the restoration of male function. Dam was a blocking factor accounting for differences in restorability among individual female seed-parents.

Source	d.f.	χ^2	*P*
Sire altitude	1	1.20	0.274
Sire region	1	2.70	0.100
Sire altitude*sire region	1	2.39	0.122
Pollination treatment	2	5.83	0.054
FA vs. FR	1	0.24	0.627
FP vs. (FA & FR)	1	5.64	0.018
Dam	31	69.77	< 0.001

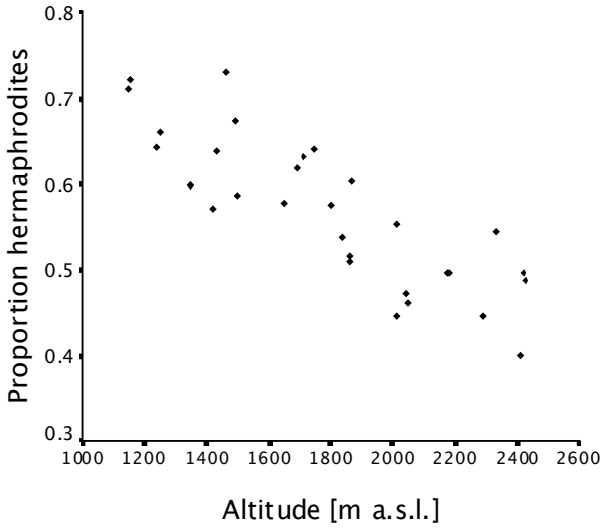

Fig. 1 Negative correlation between altitude and sex ratio among 30 late-successional populations of *Thymus praecox* agg. from five study regions in the Swiss Alps (r = -0.84, P < 0.001).

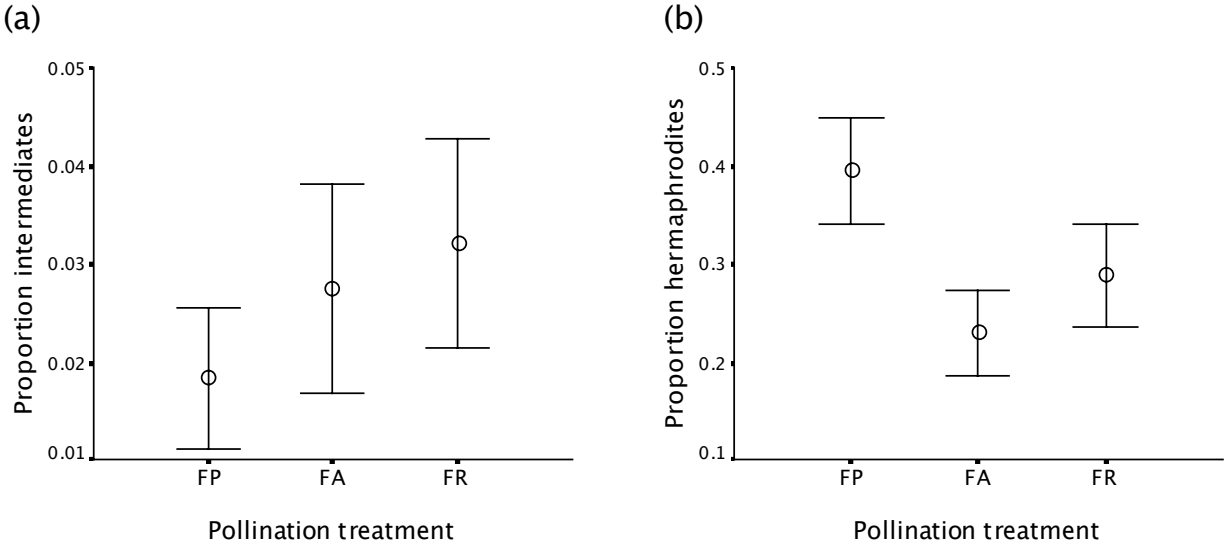

Fig. 2 Effect of cross type (FP: within population; FA: among altitude within region; FR: among region within altitude) on the proportion of intermediate sexual phenotypes (a) and the proportion of hermaphrodites (b) among 96 crosses between hermaphrodites and females of *Thymus praecox* agg. from the Swiss Alps. Data are means ± SE (n = 32 families per treatment). Note the differing scales of y-axes.

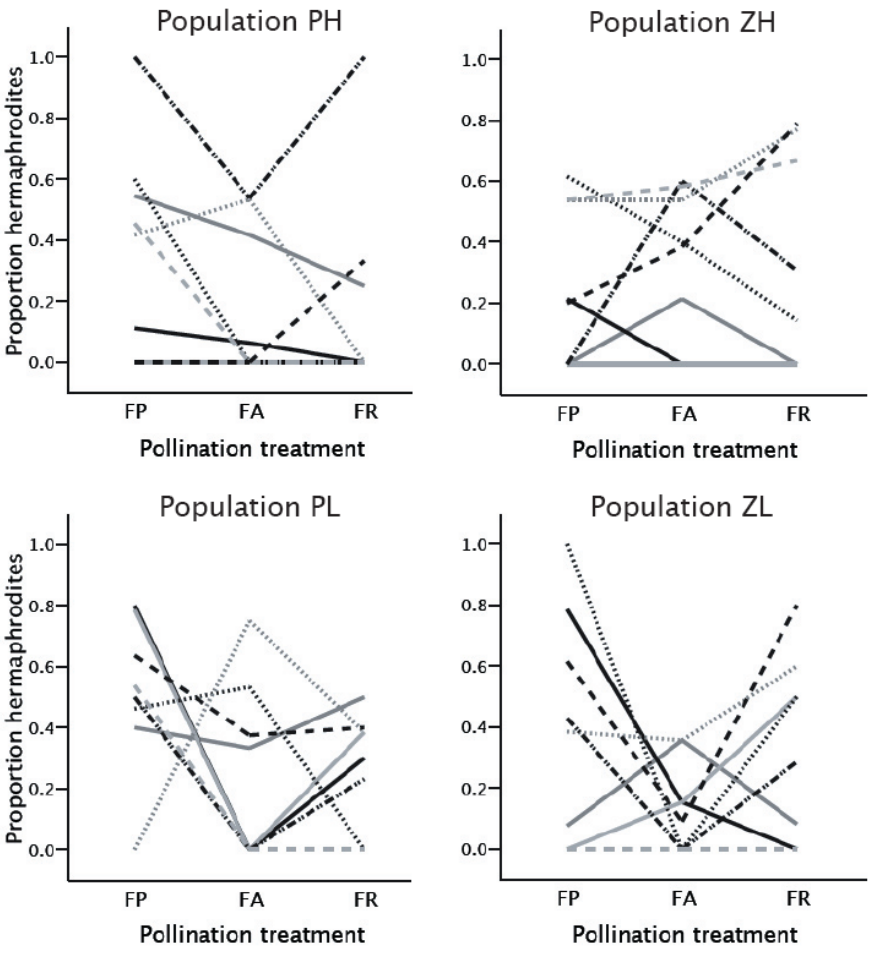

Fig. 3 Interaction plots for 32 individual females of *Thymus praecox* agg. from the Swiss Alps, grouped according to four source populations, showing their restorability in crosses with three different sires in three different pollination treatments (FP: within population; FA: among altitude within region; FR: among region within altitude).

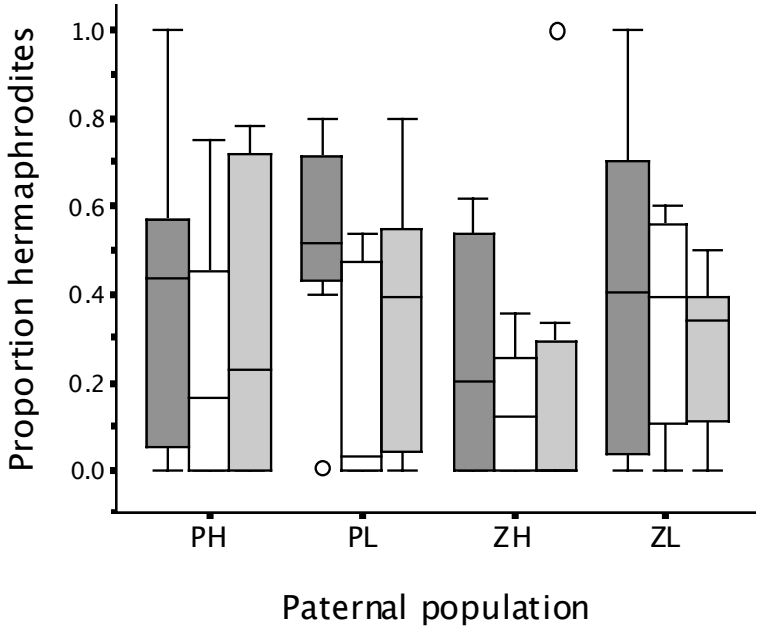

Fig. 4 Box-plots illustrating the restoration ability of four populations of sires when crossed with females from within the same population (FP, dark grey), from a different altitude within the same region (FA, white) and from a different region but similar altitude (FR, light grey) in *Thymus praecox* agg. from the Swiss Alps.

Appendix S1 Location, population abbreviation, sex ratio and ecological characterisation of 34 populations of *Thymus praecox* agg. from the Swiss Alps (see Material and Methods for details). Coordinates refer to the Swiss grid (Swiss Federal Office of Topography, Wabern, Switzerland).

			Population	Sample	Proportion		Altitude	Population		Vegetation	Mean of Landolt's ecological indicator value							
Region	Location	Coordinates	abbreviation	size	Intermediates	Hermaphrodites	[m a.s.l.]	Density	Heterogeneity	cover	F	R	N	H	D	L	T	K
Flimserstein	Fidaz-W	742560.189650	FL	106	0.047	0.642	1240	0.234	0.101	4.10	1.9	3.5	2.1	2.9	3.2	3.9	3.1	3.5
Flimserstein	Fidaz-E	742850.189750		82	0.000	0.659	1250	0.283	0.127	4.02	1.9	3.6	2.1	2.9	3.4	4.0	2.9	3.6
Flimserstein	Spaligna	740250.189860		99	0.030	0.596	1350	0.308	0.131	2.74	2.0	3.4	2.2	2.9	3.5	4.1	2.8	3.5
Flimserstein	Naraus	739550.192000		96	0.000	0.552	2010	0.250	0.102	3.68	2.4	3.5	2.3	3.0	3.1	4.0	2.2	3.2
Flimserstein	Crap da Flem	740700.192720	FH	101	0.000	0.495	2420	0.228	0.122	2.73	2.7	3.4	2.2	3.1	3.1	4.4	1.6	3.3
Flimserstein	Ils Lags	740120.192900		74	0.000	0.486	2425	0.204	0.137	3.16	2.7	3.3	2.2	3.2	3.3	4.1	1.8	3.2
Goms	Oberbach early-successional	666660.151350	GP	107	0.000	0.346	1400	na	0.115	na	1.8	3.0	2.6	2.8	3.6	3.9	3.3	3.5
Goms	Oberbach late-successional	666625.151510		88	0.000	0.625	1430	0.296	0.115	2.26	1.7	3.2	2.2	2.9	3.2	4.1	3.0	3.7
Goms	Münster	663100.149430		88	0.000	0.636	1580	0.198	0.090	3.29	2.0	2.7	2.1	3.1	3.5	3.9	2.9	3.5
Goms	Chietal	666700.153950		72	0.000	0.528	2140	0.261	0.162	3.25	2.8	2.4	2.4	3.6	3.7	3.7	2.1	2.9
Langwies	Schluocht	773070.188260		96	0.010	0.635	1430	0.217	0.110	4.38	2.1	3.6	2.1	3.1	3.8	3.9	2.8	3.4
Langwies	Matte	773650.189325	LL	104	0.000	0.615	1690	0.303	0.120	3.62	2.5	3.4	2.1	3.2	3.9	3.8	2.3	3.2
Langwies	Seta	774550.189510	LH	107	0.009	0.495	2180	0.320	0.127	4.36	2.8	3.6	2.4	3.3	3.3	3.9	2.0	3.1
Piora valley	Valle	695100.153910		66	0.000	0.576	1650	0.193	0.146	3.92	2.1	2.6	2.0	3.1	3.1	4.1	2.5	3.5
Piora valley	Piora	694960.154050	PL	89	0.011	0.629	1715	0.327	0.159	3.85	2.3	2.7	2.0	3.1	3.3	4.1	2.5	3.3
Piora valley	Alpe Ritom	694950.154900		69	0.000	0.507	1860	0.148	0.126	4.68	2.6	3.1	2.4	3.1	3.9	3.9	2.3	3.1
Piora valley	Motta	695950.155475		70	0.000	0.600	1870	0.162	0.206	4.37	2.6	3.0	2.3	3.2	3.9	4.0	2.3	3.1
Piora valley	Scüd	696550.156200		83	0.000	0.470	2040	0.160	0.154	4.83	2.5	2.5	2.2	3.4	3.9	3.8	2.3	3.2
Piora valley	Piano Corona	698670.156000		87	0.000	0.460	2050	0.246	0.130	3.88	2.7	2.4	2.5	3.4	3.8	3.8	2.1	3.0
Piora valley	Pizzo Tom	694880.155920		72	0.000	0.444	2290	0.146	0.126	3.04	2.4	3.2	2.1	2.9	3.0	4.4	1.8	3.4
Piora valley	Poncioni Negri	696050.157310	PH	100	0.000	0.400	2410	0.182	0.138	3.41	2.6	2.7	1.9	3.1	3.1	4.5	1.4	3.4
Säntis	Seealpsee	748325.237230		69	0.000	0.710	1150	0.250	0.133	3.95	2.5	3.5	2.6	3.0	3.7	3.7	2.8	3.2
Säntis	Seealp	748125.237125	SL	75	0.013	0.720	1155	0.276	0.109	3.80	2.4	3.4	2.5	3.0	3.4	3.7	2.7	3.2
Säntis	Altenalp	747500.237250		76	0.000	0.671	1490	0.211	0.137	3.96	2.1	3.5	2.3	2.9	3.4	4.0	2.5	3.4
Säntis	Weesen	748420.237810		82	0.000	0.585	1495	0.233	0.160	3.86	2.6	3.4	2.4	3.1	3.5	3.9	2.6	3.2
Säntis	Läden	747425.237840		80	0.000	0.538	1840	0.122	0.175	3.45	2.4	3.7	2.1	3.1	3.3	4.0	2.1	3.4
Säntis	Lötzlisaplsattel	746750.237190		105	0.000	0.514	1860	0.185	0.144	2.89	2.2	3.8	2.1	2.6	2.5	4.4	2.1	3.4
Säntis	Rossegg	744750.235750	SH	101	0.000	0.495	2175	0.275	0.132	3.25	2.7	3.6	2.1	3.3	3.2	4.4	1.5	3.4
Zwinglipass	Schafbergwand	745440.231650		79	0.000	0.570	1420	0.197	0.191	3.30	2.5	3.7	2.3	2.8	3.6	3.5	2.5	3.2
Zwinglipass	Tesel	746450.232075	ZL	70	0.000	0.729	1460	0.193	0.216	2.88	2.2	3.5	2.4	2.9	3.3	3.8	2.8	3.3
Zwinglipass	Chreialp	746550.232450		72	0.000	0.639	1750	0.199	0.163	3.42	2.5	3.6	2.3	3.1	3.3	4.0	2.1	3.3
Zwinglipass	Litten	746225.232575		80	0.000	0.575	1800	0.142	0.194	3.54	2.7	3.4	2.7	3.2	3.6	3.8	2.1	3.1
Zwinglipass	Zwinglipass	746825.233460	ZH	99	0.000	0.444	2010	0.216	0.179	3.77	2.6	3.5	2.1	3.1	3.0	4.3	1.6	3.3
Zwinglipass	Altmann	746020.233960		68	0.015	0.544	2330	0.144	0.130	3.66	2.8	3.7	2.0	3.0	2.9	4.3	1.5	3.2

Appendix S2 Extent and directionality of changes in sexual phenotypes of six genotypes of *Thymus praecox* agg. from the Swiss Alps that showed a labile expression of sex morph in ramets transplanted to two experimental fields, monitored across years. Out of a total of 133 randomly sampled genotypes, only two, SLH14 and ZLF05 showed labile sex. Genotype SLH14 displayed relatively small sized hermaphrodite flowers, and ZLF05 displayed relatively large sized female flowers at natural sites, as compared to other individuals of the same sex and from the same populations (U. Landergott, unpubl. data). The other four genotypes were deliberately chosen for transplanting because of their extreme flower size or intermediate sex type in natural populations. Sexual phenotypes (F = female; H = hermaphrodite; I = intermediate; nf = not flowering) of the ramets are listed and changes in the degree of restoration of the male function (+ for added restoration; – for reduced restoration) are indicated to visualise the directionality of sex change across years and locations of ramets.

	Location of ramet							
			Experimental field					
	Natural population			Low altitudinal		High altitudinal		
Genotype	Sex	Attribute	Year	Sex	Change	Sex	Change	Attribute of intermediate phenotypes
Random samples								
SLH14	H	Relatively small flowers	2003	I	-	H		Both female and hermaphrodite flowers
			2004	H		I	-	Both female and hermaphrodite flowers
ZLF05	F	Relatively large flowers	2002	I	+	F		Sparse fertile anthers
			2003	F		I	+	Sparse fertile anthers
			2004	I	+	F		Sparse fertile anthers
Labile sex expected								
PLI01	F	Conspiciously large flowers	2002	nf		nf		
			2003	F		I	+	Sparse fertile anthers
			2004	F		F		
PLI02	H	Conspiciously small flowers	2002	nf		nf		
			2003	I		I	-	Predominantly hermaphrodite flowers
			2004	I	-	I	-	Predominantly hermaphrodite flowers
PLI03	I	Predominantly female flowers	2002	nf		I		Predominantly female flowers
			2003	F	-	I		Predominantly female flowers
			2004	I		F	-	Predominantly female flowers
ZLI01	I	Predominantly female flowers	2002	nf		F	-	
			2003	F	-	F	-	
			2004	F	-	F	-	

Appendix S3 Number of flowering progenies and progeny sex ratios of 96 controlled crosses between 32 females and 32 hermaphrodites of *Thymus praecox* agg. from the Swiss Alps listed according to paternal half-sib families. Each parent was used three times, once in a within population cross (FP), once in a cross among altitudes within region (FA) and once in a cross among regions within altitude (FR).

	Cross treatment								
	FP			FA			FR		
Father	Mother	*N*	Sex ratio	Mother	*N*	Sex ratio	Mother	*N*	Sex ratio
PHH01	PHF03	9	0.11	PLF07	15	0.53	ZHF11	14	0.00
PHH02	PHF16	4	0.00	PLF05	6	0.00	ZHF14	12	0.00
PHH03	PHF11	12	0.42	PLF02	12	0.00	ZHF18	15	0.67
PHH06	PHF14	11	0.46	PLF17	10	0.00	ZHF17	13	0.31
PHH12	PHF06	12	0.00	PLF18	16	0.38	ZHF19	14	0.79
PHH15	PHF01	11	0.55	PLF13	10	0.00	ZHF08	15	0.00
PHH17	PHF07	10	0.60	PLF11	12	0.75	ZHF15	14	0.14
PHH19	PHF12	7	1.00	PLF01	12	0.33	ZHF16	13	0.77
PLH01	PLF02	5	0.80	PHF01	12	0.42	ZLF09	14	0.00
PLH04	PLF18	11	0.64	PHF14	14	0.00	ZLF05	14	0.50
PLH08	PLF11	14	0.00	PHF06	14	0.00	ZLF03	12	0.08
PLH11	PLF13	14	0.50	PHF16	13	0.00	ZLF04	13	0.00
PLH12	PLF07	13	0.46	PHF03	16	0.06	ZLF10	10	0.80
PLH14	PLF17	13	0.54	PHF11	15	0.53	ZLF06	12	0.50
PLH17	PLF01	15	0.40	PHF07	12	0.00	ZLF08	14	0.29
PLH19	PLF05	14	0.79	PHF12	13	0.54	ZLF07	15	0.60
ZHH11	ZHF14	12	0.00	ZLF04	13	0.15	PHF06	15	0.00
ZHH12	ZHF19	15	0.20	ZLF03	14	0.36	PHF16	15	0.33
ZHH14	ZHF15	13	0.62	ZLF08	14	0.00	PHF07	15	0.00
ZHH16	ZHF18	13	0.54	ZLF06	4	0.00	PHF11	11	0.00
ZHH17	ZHF08	15	0.00	ZLF09	14	0.00	PHF03	12	0.00
ZHH18	ZHF17	13	0.00	ZLF10	11	0.09	PHF14	13	0.00
ZHH19	ZHF16	13	0.54	ZLF07	14	0.36	PHF12	13	1.00
ZHH20	ZHF11	14	0.21	ZLF05	13	0.15	PHF01	4	0.25
ZLH02	ZLF07	13	0.39	ZHF15	15	0.40	PLF11	13	0.39
ZLH03	ZLF09	12	0.00	ZHF17	15	0.60	PLF07	12	0.00
ZLH05	ZLF08	14	0.43	ZHF14	15	0.00	PLF05	13	0.39
ZLH06	ZLF03	13	0.08	ZHF19	13	0.39	PLF01	14	0.50
ZLH07	ZLF06	14	1.00	ZHF18	12	0.58	PLF17	15	0.00
ZLH08	ZLF05	15	0.00	ZHF08	14	0.21	PLF02	10	0.30
ZLH09	ZLF10	13	0.62	ZHF11	15	0.00	PLF18	15	0.40
ZLH10	ZLF04	14	0.79	ZHF16	13	0.54	PLF13	13	0.23

Chapter 2

Allelic configuration and polysomic inheritance of highly variable microsatellites in tetraploid gynodioecious *Thymus praecox* agg.

Urs Landergott, Yamama Naciri, J. Jakob Schneller & Rolf Holderegger

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Abstract

Polyploidy plays a pivotal role in plant evolution. However, polyploids with polysomic inheritance have hitherto been severely underrepresented in plant population genetic studies, mainly due to a lack of appropriate molecular genetic markers. Here we report the establishment and experimental validation of six fully informative microsatellite markers in tetraploid gynodioecious *Thymus praecox* agg. Sequence data of 150 microsatellite alleles and their flanking regions revealed high variation, which may be characteristic for polyploids with a reticulate evolutionary history. Understanding the patterns of mutation (indels and substitutions) in microsatellite flanking-sequences was a prerequisite for the development of co-dominant markers for fragment analyses. Allelic segregation patterns among progeny arrays from 10 test crosses revealed tetrasomic inheritance in *T. praecox* agg. No evidence of frequent double reduction was detected. PCR based dosage effects allowed for precise assignment of allelic configuration at all six microsatellite loci. The quantification of allele copy numbers in PCR was verified by comparisons of observed and expected gametic allele frequencies and heterozygosities in test crosses. Our study illustrates how PCR based markers can provide reliable estimates of heterozygosity and, thus, powerful tools for breeding system and population genetic analyses in polyploid organisms.

Introduction

Polyploidy plays a significant role in plant evolution; a prominent example being the production of agricultural crops. Much scientific effort has been made to investigate the formation, establishment, genome organization and molecular evolution of polyploids (Bennett 2004; Soltis et al. 2004). For instance, higher selfing ability leading to better colonizing ability of polyploids relative to their diploid progenitors has been viewed to contribute to the success of polyploids (Soltis and Soltis 2000). Furthermore, population genetic theory predicts increased levels of allelic diversity and heterozygosity in polyploids (Bever and Felber 1992), which may provide a genetic buffer against inbreeding depression (Ronfort 1999; Soltis and Soltis 2000) and genetic drift (Barrett and Kohn 1991; Ronfort et al. 1998). However, empirical evidence from natural populations is still scarce and additional studies are needed to evaluate the above population genetic expectations (Ronfort 1999; Soltis and Soltis 2000; Landergott et al. 2001; Galloway et al. 2003; Lopez-Pujol et al. 2004). Empirical studies on polyploids require knowledge on their mode of inheritance (Bever and Felber 1992; Ronfort et al. 1998; Ronfort 1999). Species of presumably allopolyploid origin show disomic inheritance, where segregation is similar to that of nonhomologous pairs of chromosomes in diploids. In contrast, autopolyploids form multivalents during meiosis resulting in polysomic inheritance with a pattern of segregation varying from random assortment of homologous chromosomes to random assortment of sister chromatids. The latter phenomenon, referred to as double reduction, occurs when there is recombination between the centromere and a given locus, which increases the production of homozygous gametes (Bever and Felber 1992).
Recently, attention has been drawn on potential effects of polyploidization on the evolution of plant sexual systems via changes in self-compatibility, sex determination or inbreeding depression (Pannell et al. 2004). For example, polyploidization may trigger the evolution of dioecy in plants via the loss of self-incompatibility (Miller and Venable 2000). Moreover, as the nuclear genome becomes multiplied while the organellar genomes do not, polyploidization also affects nuclear-cytoplasmic interactions (Wendel 2000; Pannell et al. 2004). Both cytoplasmic male-sterility factors and multiple nuclear restorers of male function are involved in the sex expression in many gynodioecious plant species where female and hermaphroditic individuals coexist in the same population (Charlesworth and Laporte 1998). We are currently studying the evolution of nuclear-cytoplasmic gynodioecy in the tetraploid plant *Thymus praecox* agg. Selfing rates and inbreeding depression are important key parameters in the evolution of gynodioecious species with self-compatible hermaphrodites and obligatory outcrossed females (Jacobs and Wade 2003). Marker based estimates of selfing rates and heterozygosity are useful to infer inbreeding depression in natural populations of long-lived plants (Ritland 1990). Therefore, highly variable and co-dominant molecular genetic markers provide an important tool for investigations on the maintenance of nuclear-cytoplasmic gynodioecy in natural populations of *T. praecox* agg.

The use and interpretation of allozyme markers in polyploid species is often difficult (Hardy et al. 2001; Lopez-Pujol et al. 2004), and allelic diversity at allozyme loci is limited (Olson 1997; Galloway et al. 2003). In contrast, co-dominant microsatellite markers with high allelic diversity provide a powerful molecular genetic tool for studies on mating patterns and breeding systems. Unfortunately, in polyploid species, analysis of PCR based microsatellite markers is not as straightforward as in diploids. The quantification of the copy number per allele has often been reported as impossible in species with polysomic inheritance (Esselink et al. 2004; Nybom et al. 2004; De Silva et al. 2005; Luo et al. 2006). Recent work on roses has though highlighted the potential use of PCR based dosage effects for estimating the allelic configuration at microsatellite loci in polyploid plants via MAC-PR (microsatellite DNA allele counting-peak ratios; Esselink et al. 2004; Nybom et al. 2004). Because of varying amplification intensity among different alleles in roses, Esselink et al. (2004) and Nybom et al. (2004) analyzed all alleles in pairwise comparisons by calculating the ratios between the amplification intensities of two co-occurring alleles. MAC-PR is thus a laborious approach, and fully heterozygous individuals are desirable to determine the 1:1 ratios that are used as a base line for calculations of allele quantification in partial homozygotes. However, direct co-dominant interpretation of microsatellite loci based on relative PCR product intensities has also been reported in tetraploid sweet potato (Buteler et al. 1999), in lake sturgeon (McQuown et al. 2002), in alfalfa (Julier et al. 2003; Flajoulot et al. 2005) and in birch (Truong et al. 2005). The relatively novel interpretation of PCR based allele dosage effects should yet be done with caution in order to rule out potential artifacts (Wagner et al. 1994; Esselink et al. 2004). Both PCR selection caused by differential primer affinity and PCR drift resulting from random events during early cycles of PCR have been claimed to cause skewness in simultaneously amplified products (Wagner et al. 1994). To verify estimates of allele copy numbers, known parent-offspring relationships should be most helpful (Buteler et al. 1999; Nybom et al. 2004). Only allelic segregation patterns in conformance with expectations under a given mode of polyploid inheritance can verify allelic configuration estimates.

In the present study, we report the development, optimization and experimental validation of six fully informative and highly variable microsatellite markers in tetraploid gynodioecious *T. praecox* agg. Tetrasomic inheritance of the microsatellite markers was evident from controlled crosses. Precise tetraploid allelic configurations were directly determined from PCR based dosage effects at all six loci. Mendelian segregation of the microsatellite alleles confirmed the quantification of allele copy numbers in PCR. Heterozygosity was unambiguously scored in individuals from geographically remote natural populations of *T. praecox* agg. From a general viewpoint, our work outlines access to fully informative PCR based molecular markers representing a powerful tool for breeding system and population genetic analyses in polyploid species.

Materials and methods

Study species

Hybridization is very common in the genus *Thymus* (Lamiaceae), especially among tetraploid taxa (Jalas and Kaleva 1970; Stahl-Biskup and Sáez 2002). In the phylogenetically young section *Serpyllum*, the central European tetraploid representatives are morphologically highly diverse and taxonomically difficult. Jalas and Kaleva (1970) hypothesized that each of these tetraploid taxa probably originated from multiple allopolyploidization events between several diploid species. Taxonomists have classified the *Thymus* taxa of the European Alps in a wealth of subspecies, varieties and formae. In a more sensible approach, Jalas (1970) suggested to treat several tetraploid taxa of the European mountains as members of the single polymorphic species aggregate *Thymus praecox* Opiz ampl. Jalas, with five subspecies (Tutin et al. 1972). Our study populations are best assigned to *T. praecox* Opiz ssp. *polytrichus* (A. Kerner ex Borbás) Jalas. However, as the subspecific boundaries are vague (Jalas 1970, 1971), we will only refer to *T. praecox* agg. in the following. Individuals of gynodioecious *T. praecox* agg. are long-lived and form large cushions. Hermaphrodites are self-compatible. The species is widespread in the European Alps from subalpine to alpine altitudes and is widely found on rocky surfaces and in pastures.

The individuals of *T. praecox* agg. used in the present work originated from study populations in the Swiss Alps from the geographically remote regions Flimserstein [F], Goms [G], Langwies [L], Piora valley [P], Säntis [S] and Zwinglipass [Z], from both low [L] and high [H] altitudinal sites each. Precise locations are available from the authors upon request. Individuals were labeled by sex [F = female; H = hermaphrodite] and identified by a number per sexual phenotype. Individual PLF05 thus refers to the study female number five from a low altitudinal population in the Piora valley. For permanently marked females in natural populations, seeds from open pollination were collected and grown in a greenhouse. From a controlled crossing experiment of individuals transplanted to two experimental fields, full-sib families were available to investigate the mode of inheritance in *T. praecox* agg. (see below for sample sizes).

Additionally, two individuals from one population of each of four closely related *Thymus* taxa were collected: the tetraploid *T. praecox* Opiz ssp. *arcticus* (E. Durand) Jalas from Ireland, the diploids *T. pulegioides* L. from the Swiss Alps and *T. serpyllum* L. from Hungary (all section *Serpyllum*) and the Mediterranean diploid *T. vulgaris* L. (section *Thymus*) from France.

Development of microsatellite markers

We isolated microsatellite loci in *T. praecox* agg. from a partial genomic library, screened on nylon membranes following the procedure of Estoup and Martin (1996). From two individuals of *T. praecox* agg., genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), further purified with the Prep-A-Gene Purification System (Biorad) and pooled for digestion

with *Alu*I, *Hae*III and *Rsa*I (Boehringer Mannheim). Size-selected fragments (500-1000 bp) were ligated in pUC18, and ultra-competent cells (Stratagene) were transformed. A total of 2500 recombinant clones were transferred to LB-agar plates and consecutively lifted onto nylon membranes (Boehringer Mannheim). Hybridization was carried out with digoxigenin-labeled probes [$(AC)_{12}$, $(AG)_{12}$, $(AAT)_8$ and $(TCT)_8$], prepared with the DIG oligonucleotide tailing kit (Roche Molecular). A total of 19 hybridizing clones were detected with the DIG Luminescent Detection Kit (Roche Molecular) and used as PCR templates for sequencing with the universal pUC/M13 primers.

Different microsatellite repeat motifs were found in 12 sequences (0.5% of the recombinant clones; GeneBank accession nos. AM087131-AM087142) and primers were designed for sites in the flanking regions suitable for PCR amplification. Amplifications were carried out in 10µl reaction volumes (see below for optimized reaction conditions). The PCR products were separated on Spreadex gels (EL 300-600, Elchrom Scientific), stained with ethidium bromide and recorded under UV light. Initial amplification products of microsatellite loci in *T. praecox* agg. pointed to three main problems that needed to be addressed for further optimization of the markers: (1) The performance in PCR varied markedly among individuals suggesting differences in the quality of template DNA; (2) the magnitude of size differences of alleles suggested the presence of size variation in the microsatellite flanking regions; (3) there was evidence of null alleles due to mutations within primer sites.

A series of DNA extractions testing different protocols and additional precipitation and purification reagents was carried out in order to improve DNA quality (thyme is well known for its various secondary metabolites and intraspecific chemical polymorphism; Stahl-Biskup and Sáez 2002). Best results were obtained from the DNeasy Plant Mini Kit (Qiagen) with the following modifications to the manufacturer's protocol: A maximum of 10 mg of silica dried leaf materiel was used. In the protein precipitation step, 20µl of 25% PVP (polyvinyl-pyrrolidone) was added in order to remove phenolic compounds. After the binding of DNA to the minicolumn, it was washed twice with 600µl of a desalting wash buffer (400 mM NaCl, 20 mM Tris (pH 7.5), 2 mM EDTA (pH 7.5) and 50% EtOH (v/v); Prep-A-Gene Purification System, Biorad) and once with 500µl of 80% EtOH. Extracted DNA was stored in Tris-buffer.

In order to isolate alleles of microsatellites together with their adjacent flanking regions for sequencing, gel pieces of well-separated bands were picked from Spreadex gels under UV light using 1000µl pipette tips. Gel pieces were ejected into 10µl TE, incubated over night and 1µl of the resulting solution was used as template for direct re-amplification of isolated alleles in 15µl reaction volumes. Re-amplification products were purified with the Prep-A-Gene Purification System (Biorad) and sequenced in both directions using BigDye™ Terminator Ready Reaction Kit 2.0 on an ABI 3100 automated sequencer (Applied Biosystems). Sequencing signals tended to decrease or, in some cases, broke down after the microsatellite repeat array. Sequences were aligned manually in SEQUENCHER (version 3.1.1; Gene Codes Corporation). New, mostly degenerate primers were designed for conservative sites close enough to the microsatellite locus in order to exclude insertions/deletions in the flanking

regions. Since ghost bands on nondenaturing Spreadex gels did not allow an unambiguous interpretation of tetraploid allelic patterns, HPLC purified labeled primers (6FAM and HEX, Microsynth; NED, Applied Biosystems) were used to score the most promising loci on an ABI 3100 automated sequencer (36 cm capillaries, POP-6, Rox 400 HD internal size standard, GENESCAN version 3.7; Applied Biosystems). Electropherogram peak heights were used to estimate the copy number per allele. PCR conditions (annealing temperature, annealing and extension times, as well as concentrations of primers, $MgCl_2$ and BSA) were optimized for best detection of allelic configurations. As several locus-individual combinations still failed to resolve tetraploid allelic configuration (null alleles), additional alleles were isolated using more remote primers in combination with degenerate ones located close to the microsatellite. Alleles were sequenced from the remote primer only. After sequence alignment, new, even higher degenerate primers were designed (Table 1; Supplementary Material I).

Optimized PCR amplifications were carried out using 1 ng of template DNA in 10 µl reaction volumes of 0.15 mM of each dNTP, 5% DMSO, 1× polymerase buffer, 0.025 U/µl *Taq* DNA polymerase (Sigma) and locus-specific concentrations of primers, $MgCl_2$ and BSA (Table 1). Cycles were run on Genius and TC-412 thermal cyclers (Techne) with initial denaturation for 3 min at 96°C, locus-specific (Table 1) number of cycles of 35 s at 95°C, locus-specific annealing time and temperature, locus-specific elongation time at 72°C and final extension of 20 min at 60°C. The cooling ramp was set to 0.5°C/s, starting from 65°C for loci D257 and D347 and from 60°C for the remaining four loci. Loci D346 and D347 had the most degenerate primers (Table 1) with considerably different optimal annealing temperatures predicted per oligonucleotide variant (Microsynth). Therefore, these two loci were also tested for improved amplification performance with the Multiplex PCR Kit (Qiagen). This PCR Master mix contains a synthetic factor MP that stabilizes specifically bound primers and enables efficient extension of all primers in a reaction. We added DMSO and BSA as above to 10 µl reaction volumes. Initial denaturation of 15 min at 95°C was applied to activate the HotStarTaq DNA polymerase. Clean amplification products at decreased annealing temperature and improved estimates of allelic dosage were obtained for locus D347 for which the Multiplex PCR Kit was chosen as the standard protocol (Table 1).

Inheritance in controlled crosses, confirmation of allelic configuration estimates and resolution power of the microsatellite markers

To investigate segregation patterns at the six microsatellite loci, we used seedlings of 10 test crosses (Table 2): Two hermaphrodites from two different populations were (1) selfed (crosses A and F), (2) outcrossed with a hermaphrodite from within the same population (B and G) and (3) used as a pollen donor in a within population cross with a female (C and H). The two females were additionally crossed (4) with hermaphrodites from a different population within study region P (D and I) and (5) from the geographically remote region Z (E and K). Our crossing design provided four large half-sib families, those of the parental individuals PHH06, PHF14, PLH19 and PLF05, which were used in three different crosses each (Table 2). A total

of 75 progenies were genotyped. One seedling of cross A proved to be outcrossed with an unknown father and was therefore excluded, and one seedling of cross B was a self and thus included in cross A for analyses.

Maternal and paternal gamete genotypes were determined for each progeny and each locus from the allelic composition of parental genotypes (Supplementary Material II). The mode of inheritance (disomic *vs*. polysomic) was inferred from the pairing pattern of parental alleles among gametes. Under polysomic inheritance, the formation of a homozygous gamete from a single copy parental allele would provide direct evidence for double reduction. In a larger data set including all the parental alleles, excess gamete homozygosity would provide indirect evidence for double reduction.

In order to check the accuracy of the quantification of allele copy number in PCR, we recorded the allele frequencies among all gametes per parental individual and locus (including frequencies among progenies derived from self pollination even though the genotypes of the gametic phase were not known in these families). These observed gametic allele frequencies were tested for significant departure from those expected from parental allelic configurations under random chromosome segregation by means of Chi-squared tests (Table 3). Furthermore, for each parental individual and locus, we recorded the observed frequency and calculated the expected frequencies of homozygous gametes under both random chromosome and random chromatid segregation (with a maximal frequency of double reduction of 1/7 in the latter case; Bever and Felber 1992). Wilcoxon signed-ranks tests were used to test for systematic deviation of observed from expected frequencies of homozygous gametes per locus.

To check the applicability of the microsatellite markers to geographically remote samples of *T. praecox* agg., 10 females, one each of a low and high altitudinal population from regions F, L, P, S and Z were genotyped. Per female, two offspring of open pollinated origin (= obligatory outcrossed) were genotyped. This also permitted to assess the efficiency of the markers in resolving outcrossing events in natural populations. The relationship between the number of different alleles per locus and both the number of detected outcrosses and the mean observed heterozygosity (Table 1) was studied with Spearman rank correlation coefficients. Observed individual heterozygosity was scored following Bever and Felber (1992), with the genotypes weighted by 1 minus the probability of any two alleles being identical by descent (*AAAA* = 0, *AAAB* = 0.5, *AABB* = 0.667, *AABC* = 0.833 and *ABCD* = 1). Statistical analyses were performed in SPSS (version 10.0.8 for Mac; SPSS Inc.).

Results

Sequence variation in flanking regions and within microsatellite repeat arrays

A total of 150 alleles out of six microsatellite loci of *T. praecox* agg. was sequenced. These alleles were isolated from 9-20 individuals per locus, issued from 11 geographically remote natural populations of *T. praecox* agg. Full sequences of alleles are listed in Supplementary

Material I, along with adjacent flanking-sequences. The sequence characterization of the six loci revealed high variation both in flanking regions and in microsatellite repeat arrays.

Within microsatellite flanking regions, substitutions as well as indels (insertions/deletions) were frequent at all six loci. In several loci, one flanking region was relatively conservative while the other was variable. Different flanking-sequence variants were detected within and among individuals of *T. praecox* agg., and particular variants were repeatedly found in samples from geographically remote regions (e.g. an indel mutation in locus D257 and a series of base substitutions in loci C405 and E070). As expected from the commonly high mutation rate within repeat arrays (Dettman and Taylor 2004), different flanking-sequence variants were associated with multiple repeat alleles. However, divergent flanking-sequence variants also shared identical repeat alleles (e.g. in loci C405, E070 and E089). Primer pairs could be designed to exclude flanking-sequence indels from marker fragments for three loci. However, immediately adjacent to the dinucleotide repeat microsatellites C405 and E070, short deletions of an even number of bases were detected confusing variation in repeat number in fragment analyses. In locus D347, several indels remained in the marker fragment. Base substitutions within flanking regions were so frequent that most of the final primers designed for microsatellite marker fragment amplification had to be degenerate (Table 1).

Within microsatellite arrays, mutations other than changes in the number of repeat units were common. An insertion caused odd numbers of bp in a part of the alleles of the dinucleotide microsatellite E070. Indels were also found in microsatellite D347. Base substitutions within repeat arrays were detected in the microsatellites D346, D347 and, most prominently, in locus D257. Furthermore, the duplication of repeats with substitutions (imperfect repeats) was observed in locus D257. Out of a total of 29 repeat alleles sequenced from locus D257, the number of different lengths was 12, but the number of unique sequences was 23.

Allele size diversity and resolving power of the microsatellite markers

Allelic diversity based on marker fragment length was determined for each of the six microsatellite loci in 40 individuals from 10 natural populations of *T. praecox* agg. (Table 1). The trinucleotide microsatellite E089 showed the lowest variation in repeat number with a total of six different alleles. The four dinucleotide repeat loci C405, D257, D346 and E070 displayed 17-24 different alleles, and the compound microsatellite D347 showed the highest variation in repeat number with 39 different alleles in the 40 individuals studied. The number of different alleles per locus was positively correlated with its power to resolve outcrossing events in natural populations ($r_s = 0.986$, $P = 0.000$; Table 1). All of the 10 females and their 20 obligatory outcrossed offspring from different populations were genetically distinguished by means of the single, highly polymorphic locus D347. The same result was obtained from the combined analysis of the four dinucleotide repeat loci. These highly variable microsatellite

markers are thus valuable to investigate selfing rates of hermaphrodites in natural populations of *T. praecox* agg.

Inheritance of microsatellite markers

Parental alleles were randomly combined in gamete genotypes at all six microsatellite loci as inferred from 10 test crosses (e.g. in male gametes of individual ZHH18 at locus E089; Supplementary Material II). This showed that inheritance in *T. praecox* agg. is tetrasomic. No direct evidence for double reduction was detected at any of the six loci, based on observations of heterozygous gametes (C405: $n = 93$; D257: $n = 86$; D346: $n = 108$; D347: $n = 116$; E070: $n = 95$; E089: $n = 36$). Furthermore, at all of the six loci, heterozygosity among gametes was significantly higher than expected under maximal double reduction (i.e. under random chromatid segregation; Table 3).

Assignment of microsatellite allelic configurations: heterozygosity estimates

Direct determination of tetraploid allelic configurations from PCR product intensities was possible for all six microsatellite loci and almost all of the 114 individuals of *T. praecox* agg. genotyped in the current study (Figure 1). Electropherogram peak heights of the six microsatellite markers in the 84 individuals analyzed from controlled crosses are listed in Supplementary Material II. Best homogeneity among amplification product intensities was obtained by applying annealing temperatures from the lower boundaries of those predicted for the different variants of a degenerate primer pair (except for locus D257), by adjusting the primer concentrations for the degree of degeneration and by optimization of annealing time (Table 1). Longer repeat alleles tended to display lower amplification intensities due to kinetic effects of PCR (e.g. in the progeny array of cross K at the loci D257 and E070; Supplementary Material II). Kinetic effects could be reduced by optimization of extension times and of BSA- and $MgCl_2$-concentrations (Table 1). Increased concentrations of BSA and $MgCl_2$, however, did also increase the amount of stutter products.

Stutter fragments, one and two repeat units shorter than the actual allele, were more pronounced the higher the repeat number was (Figure 1). Therefore, stutter peak heights were added up to allele peak heights in order to estimate allelic dosage in loci C405, D257, D346 and D347 (Supplementary Material II). Corrections for the slope of kinetic effects did further improve estimates of allelic dosage (data not shown), but such calculations were not necessary for the assignment of allelic configurations in the present data set. Some differential amplification intensity among alleles was detected at the three loci D257, D346 and D347 (Supplementary Material II). Allelic differences in amplification intensity were consistent within individuals and among their offspring. However, amplification intensity could vary within allele size among individuals. For example, allele D257-*g* was under-amplified in individual PHH02, but not so in PHH01 (Supplementary Material II). Similarly, allele D346-*d* was overamplified in PHH06, but not so in PHF14, and allele D346-*a* was underamplified in PLH04 and PLH19, but less so in PLF05 and PLH08. The MAC-PR approach was needed for

locus D346 to assign the allelic configuration of individual D05. A weak erroneous product of 98 bp in marker D346 also co-amplified in some individuals (Supplementary Material II) and needed to be taken into account for the assignment of allelic configuration (e.g. in individuals PHF14 and C02; Figure 1).

The analysis of gamete genotypes as inferred from the test crosses confirmed that the quantification of allele copy numbers in parental individuals was accurate. Allele frequencies among gametes were in good congruence with those predicted from parental allelic configurations at all of the six microsatellite loci (except for PHH06 for locus C405; Table 3). The observed heterozygosity among gametes was not significantly different from that expected under random chromosome segregation at any of the six loci (Table 3).

High levels of heterozygosity were observed among the 40 individuals from natural populations of *T. praecox* agg. (Table 1). Mean observed heterozygosity was positively correlated with allele diversity per locus ($r_s = 0.868$, $P = 0.025$). Full heterozygotes were frequently detected at all loci other than E089. The fully homozygous state was found only in one individual for locus D257 and in six individuals for locus E089. Only in three individuals, evidence of null alleles was observed (locus D346 in individual ZHH18; locus D347 in PLH19; locus E070 in an offspring of SLF08). Controlled crosses revealed an average reduction in observed heterozygosity of 0.20 in the progenies derived from selfing of hermaphrodites PHH06 and PLH19 as compared to their maternal half-sibs derived from outcrossing (Table 2, Figure 1). The observed loss of heterozygosity due to inbreeding was close to the expected value of 0.17 per generation (under random chromosome segregation; Bever and Felber 1992).

Cross-amplification in related thyme species

All of the six markers successfully and consistently amplified microsatellite loci in *T. praecox* ssp. *arcticus*, *T. pulegioides*, *T. serpyllum* and *T. vulgaris* (except for E089 in the latter species). Allelic configurations were in conformance with the ploidy levels of these thyme species (Tutin et al. 1972). Detected allele sizes were in the range of those found in *T. praecox* agg.

Discussion

The development of fully informative microsatellite markers in tetraploid *T. praecox* agg. had two major technical obstacles, which had their cause in evolutionary features of polyploid plant species. First, *T. praecox* agg. showed polysomic inheritance. Fully informative genetic markers should thus reliably estimate allele copy number at a given locus. Tetrasomic inheritance points to an autopolyploid origin of *T. praecox* agg. In contrast, Jalas and Kaleva (1970) supposed an allopolyploid origin of the species based on morphological variation. Independent of the type of polyploidization, however, recurrent formation and reticulate evolution seem to be the rule rather than the exception in polyploid plant species (Soltis et al.

2004). Not unexpectedly, therefore, high genetic diversity in microsatellite loci and flanking regions was a second major characteristic complicating the establishment of microsatellite markers in *T. praecox* agg.

High variation in microsatellite flanking-sequences and within repeat arrays

Flanking regions of microsatellites have been reported to show mutation rates of an order of magnitude lower than those found within microsatellite repeat arrays (Dettman and Taylor 2004). The variation in microsatellite flanking regions may thus be useful to infer phylogenetic relationships among closely related species (Rossetto et al. 2002; Dettman and Taylor 2004). However, we detected high within-species variation in microsatellite flanking regions in *T. praecox* agg., and geographically remote populations shared divergent flanking-sequence variants. Our findings may be characteristic for a polyploid species with a reticulate evolutionary history. The successful cross-amplification of the six microsatellite markers in related thyme species indicates that *T. praecox* agg. could have accumulated different flanking-sequence variants present in the genus. The accumulation of sequence diversity may further point to multiple origins of the tetraploid aggregate species and/or to introgression from other tetraploid thyme species. Our molecular data are in agreement with reports on extraordinarily high morphological variation and a mixing of otherwise either southerly or northerly distributed biochemical compounds in *T. praecox* agg. from the European Alps (Jalas 1970; Jalas and Kaleva 1970; Bischof-Deichnik et al. 2000).

Technically, indel mutations in flanking regions should be excluded from marker fragments, because they can cause differences in fragment length that are not attributable to differences in repeat number at the microsatellite. Additionally, base substitutions within primer sites can cause amplification failure and null alleles.

Understanding the patterns of mutation within hypervariable microsatellite loci is crucial for a biologically meaningful interpretation of markers (Balloux and Lugon-Moulin 2002; Dettman and Taylor 2004). Sequence data provided evidence for multiple factors causing marker allele size homoplasy in *T. praecox* agg. (Supplementary Material I). (1) In several loci, the association of repeat alleles of identical state with different flanking-sequence variants indicated that it was rather unlikely that all copies of the same repeat allele were identical by descent. (2) Indels in the flanking-sequence next to the microsatellite repeat array could not be avoided for three loci (C405, D347 and E070). (3) Substitution mutations within repeat arrays caused size homoplasy at three loci (D257, D346 and D347). Allele size homoplasy would seriously interfere with estimates of genetic differentiation (Balloux and Lugon-Moulin 2002) and leads to underestimation of actual heterozygosity. High allele size diversity at microsatellite loci (e.g. D257 and D347) will in turn decrease the probability for homoplasy within individuals. Indeed, the high levels of heterozygosity observed in individuals from natural populations of *T. praecox* agg. suggested that underestimation of heterozygosity should be of a minor magnitude. Both microsatellite allele size diversities and observed heterozygosities were markedly higher in tetraploid *T. praecox* agg. (Table 1) than those

reported in polyploid *Rosa* sect. *Caninae* (Nybom et al. 2004) and *Betula pubescens* ssp. *tortuosa* (Truong et al. 2005).

Our findings corroborate recommendations to sequence microsatellite alleles of a representative subsample of individuals in order to assess their suitability for addressing particular questions (Buteler et al. 1999; Dettman and Taylor 2004). We directly isolated microsatellite alleles from high-resolving electrophoretic gels for subsequent sequencing. This approach was efficient to assess the patterns of mutation in microsatellite loci and their flanking-sequences for our purpose, i.e. for the design of markers that should reliably estimate relative levels of heterozygosity among individuals.

Allelic configuration and inheritance of microsatellites

The high variability of microsatellites facilitated the determination of putative gamete genotypes at the six study loci for most of the 10 test crosses. The random combination of parental alleles in gametes rejected disomic inheritance and demonstrated tetrasomic inheritance in *T. praecox* agg. Random chromosome and random chromatid segregation are endpoints of a continuum in polysomic inheritance and most loci probably fall somewhere between these two extremes (Bever and Felber 1992). We did not attempt to obtain precise estimates of double reduction in the current study. Larger sample sizes would be required for this purpose as well as knowledge on selective forces possibly affecting heterozygosity in a gynodioecious species (Bailey et al. 2003; Hansson and Westerberg 2002). However, the results from controlled crosses indicated that the frequency of double reduction was, at best, low at all of the six microsatellite loci, i.e. that inheritance was close to random chromosome segregation.

Under polysomic inheritance, various allelic configurations and heterozygosity states are possible at a given locus (Bever and Felber 1992). Thus, the fundamental prerequisite for the assignment of microsatellite allelic configurations in a polyploid species with polysomic inheritance is the accurate reproduction of allele copy number by PCR. It has been hypothesized, however, that amplification bias may occur among simultaneously amplified fragments due to PCR drift and/or PCR selection, which would impede the interpretation of PCR based dosage effects (Wagner et al. 1994). Full repeatability of relative allele peak intensities between independent PCRs ruled out the occurrence of random PCR drift in our amplifications. In contrast, differential amplification intensities among alleles provided evidence for PCR selection resulting from differential affinity of degenerate primer variants. These effects of PCR selection could largely be reduced by optimization of PCR conditions.

Most recently, MAC-PR has been proposed as an alternative approach to deal with differential amplification intensities among alleles in polyploid plant species (Esselink et al. 2004). A basic assumption of the MAC-PR method is the repeatability of relative allelic amplification intensities among individuals and, thus, homology of microsatellite marker alleles within a species. Sequence data highlighted that this assumption is likely violated in *T. praecox* agg. due to potential repeat allele size homoplasy. Indeed, for the few alleles

showing skewed amplification intensity, skewness markedly varied among individuals. Therefore, MAC-PR may be useful to improve determination of allelic configuration within crossing families, but it would not be generally applicable for estimating allelic dosage in screening natural populations of *T. praecox* agg.

The validation of co-dominant molecular markers finally comprises the conformance of allelic segregation patterns with those predicted under a certain mode of inheritance, which is tetrasomic with nearly random chromosome segregation in the case of *T. praecox* agg. No major departure from Mendelian tetrasomic inheritance with random chromosome segregation was detected in allelic segregation patterns among the six microsatellite loci studied. Thus, the set of markers yields reliable estimates of heterozygosity at these microsatellite loci and is, despite the minor drawbacks mentioned above, appropriate to address evolutionary questions in *T. praecox* agg.

Conclusions

Polyploids have hitherto been largely neglected in plant population genetic studies, mainly due to a lack of suitably variable and fully informative molecular genetic markers (Esselink et al. 2004; Soltis et al. 2004). Our present study demonstrates that PCR techniques are appropriate to determine allele copy numbers under polysomic inheritance. Knowledge on sequence variation in microsatellite loci and their flanking regions was though a prerequisite for the establishment of co-dominant microsatellite markers in tetraploid *T. praecox agg*. High levels of variation other than changes in the number of repeat units should generally be expected in microsatellites of species with a reticulate evolutionary history, i.e. in many polyploid plants.

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Table 1 Characterisation, amplification conditions and allele diversity of six microsatellite markers in *Thymus praecox* agg.

Locus	Primer	Label	Primer sequence (5')	Primer [µM]	Annealing Temperature	Annealing Time	Elongation time	$MgCl_2$ [mM]	BSA [µg]	No. of cycles	Repeat	Fragment size (bp)[a]	No. of alleles[a]	H_O[a]	Detected outcrosses[b]
C405	F1	6FAM	GGTGAAGATGGGATTTGCTAC	0.2	50.0°	65"	14"	2.0	5.0	25	CT	148-184	19	0.92	17
	R4		GASGATAAGACTTYMGTAMAASC	0.8											
D257	F3b		TCTCCRAASMCCCAAACTATTCC	0.6	57.5°	85"	06"	1.5	5.0	26	AG	73-142	24	0.90	19
	R2	HEX	CGATCCATGGCTTGACATGTGC	0.2											
D346	F4	6FAM	CATMAWCCCCATAAWAGCARCAC	1.2	46.0°	95"	07"	1.5	5.0	25	AG	82-146	24	0.92	18
	R2d		CTCTCAAAMACACAYACCAASC	0.9											
D347	F3b		YACACACACAGYGDAGGTG	1.1	51.0°	85"	14"	3.0	5.0	26	(GA)(GAA)	104-163	39	0.96	20
	R2b	NED	GTGCCTCCYTCTATWCATCAC	0.7											
E070	F4	HEX	CCCATGTTGYAGTTACTGCG	0.4	46.0°	70"	10"	2.0	7.5	26	GA	125-155	17	0.83	16
	R6		GCCATTTTCATYYMTMTCTCC	0.9											
E089	F3		AARAGAAGAGRAGAAGAA	0.7	46.0°	65"	08"	2.0	5.0	24	GAA	124-142	6	0.54	9
	R4	6FAM	GAAGAAGTAAACCTGAAATCC	0.2											

H_O, observed heterozygosity

[a]Based on fragment analyses of 40 partly related individuals from natural populations (10 females with two offspring each; 10 parental individuals of controlled crosses).

[b]Out of 20 obligatorily outcrossed offspring of 10 females from natural populations.

Table 2 Controlled crosses and estimates of multilocus heterozygosity within progeny arrays of *Thymus praecox* agg.

Cross	Mother × father	Treatment	Progenies[a]	MLH_O[b]
A	PHH06 × PHH06	selfed	8	0.591
B	PHH06 × PHH01	outcrossed within population	6	0.744
C	PHF14 × PHH06	outcrossed within population	8	0.783
D	PHF14 × PLH04	outcrossed among populations within region	8	0.717
E	PHF14 × ZHH18	outcrossed among regions	8	0.854
F	PLH19 × PLH19	selfed	8	0.642
G	PLH19 × PLH08	outcrossed within population	8	0.800
H	PLF05 × PLH19	outcrossed within population	8	0.750
I	PLF05 × PHH02	outcrossed among populations within region	6	0.811
K	PLF05 × ZLH05	outcrossed among regions	6	0.889

[a]Number of progenies genotyped per family.

[b]Estimate of multilocus herterozygosity calculated from observed heterozygosities at five microsatellite loci (locus D347 was excluded due to missing data caused by a null allele; Table 3).

Table 3 Comparison of observed and expected gametic allele frequencies and heterozygosities at six microsatellite loci in 10 controlled crossing families of *Thymus praecox* agg.

			Gametes inherited both via pollen and ovules								
							Homozygous				
	Parent		Allele frequencies[a]			Ascertained	RCeS			RCdS	
Locus	Individual	Genotype	Expected (RCeS)	Observed	$P(\chi^2)$[b]	genotypes[c]	O	E	$P(Z)$[d]	E	$P(Z)$[d]
C405	PHH06	*ccel*	22c-11e-11l	19c-7e-18l	0.042	8	1	1.3		2.3	
	PHH01	*acde*				6	0	0.0		0.9	
	PHF14	*ackm*	10a-10c-10k-10m	14a-7c-11k-8m	0.392	20	0	0.0		2.9	
	PLH04	*aaac*									
	ZHH18	*iijj*	8i-8j	9i-7j		8	3	2.7		3.4	
	PLH19	*aade*	32a-16d-16e	30a-15d-19e	0.687	15	1	2.5		4.3	
	PLH08	*aacj*	8a-4c-4j	11a-2c-3j		8	3	1.3		2.3	
	PLF05	*cefi*	10c-10e-10f-10i	9c-11e-12f-8i	0.801	20	0	0.0		2.9	
	PHH02	*bcgj*	3b-3c-3g-3j	2b-4c-2g-4j		6	0	0.0		0.9	
	ZLH05	*abhj*	3a-3b-3h-3j	4a-4b-2h-2j		6	0	0.0		0.9	
						97	8	7.8	0.854	20.6	0.015
D257	PHH06	*accj*	14a-28c-14j	15a-30c-11j	0.651	8	0	1.3		2.3	
	PHH01	*ccgj*									
	PHF14	*ccfi*	23.5c-11.75f-11.75i	25c-8f-14i	0.422	24	5	4.0		6.9	
	PLH04	*ackl*	4a-4c-4k-4l	4a-3c-4k-5l		8	0	0.0		1.1	
	ZHH18	*eefi*	7.5e-3.75f-3.75i	8e-3f-4i		8	1	1.3		2.3	
	PLH19	*accf*	12a-24c-12f	14a-24c-10f	0.717	8	0	1.3		2.3	
	PLH08	*bcdh*	4b-4c-4d-4h	3b-5c-4d-4h		8	0	0.0		1.1	
	PLF05	*aacc*	12a-12c	10a-14c	0.414	12	4	4.0		5.1	
	PHH02	*adgm*	3a-3d-3g-3m	4a-2d-1g-5m		6	0	0.0		0.9	
	ZLH05	*bekn*	3b-3e-3k-3n	2b-4e-4k-2n		6	0	0.0		0.9	
						88	10	12.0	0.269	22.9	0.007
D346	PHH06	*cdem*	14c-14d-14e-14m	11c-14d-18e-13m	0.603	14	0	0.0		2.0	
	PHH01	*bbeg*	6b-3e-3g	7b-2e-3g		6	1	1.0		1.7	
	PHF14	*acde*				24	0	0.0		3.4	
	PLH04	*aaeh*				8	2	1.3		2.3	
	ZHH18	*bdf‡*	4b-4d-4f-4‡	4b-6d-3f-3‡		8	0	0.0		1.1	
	PLH19	*aijn*	13a-13i-13j-13n	7a-15i-11j-19n	0.104	16	0	0.0		2.3	
	PLH08	*abjk*				8	0	0.0		1.1	
	PLF05	*ajjo*	6a-12j-6o	5a-13j-6o	0.882	12	2	2.0		3.4	
	PHH02	*abfl*	3a-3b-3f-3l	4a-3b-3f-2l		6	0	0.0		0.9	
	ZLH05	*befh*	3b-3e-3f-3h	4b-4e-3f-1h		6	0	0.0		0.9	
						108	5	4.3	0.317	19.1	0.005

Table 3 continued

			Gametes inherited both via pollen and ovules								
							Homozygous				
	Parent		Allele frequencies[a]			Ascertained	RCeS			RCdS	
Locus	Individual	Genotype	Expected (RCeS)	Observed	$P(\chi^2)$[b]	genotypes[c]	O	E	$P(Z)$[d]	E	$P(Z)$[d]
D347	PHH06	*flnp*	15f-15l-15n-15p	15f-21l-15n-9p	0.187	14	0	0.0		2.0	
	PHH01	*dkpu*	3d-3k-3p-3u	4d-1k-5p-2u		6	0	0.0		0.9	
	PHF14	*joux*	12j-12o-12u-12x	10j-13o-11u-14x	0.841	24	0	0.0		3.4	
	PLH04	*dhty*	4d-4h-4t-4y	2d-5h-5t-4y		8	0	0.0		1.1	
	ZHH18	*cemv*	4c-4e-4m-4v	4c-5e-4m-3v		8	0	0.0		1.1	
	PLH19	*gmn‡*	13.8g-13.8m-13.8n-13.8‡	13g-20m-8n-14‡	0.152	16	0	0.0		2.3	
	PLH08	*irsw*	4i-4r-4s-4w	4i-4r-6s-2w		8	0	0.0		1.1	
	PLF05	*flps*	10f-10l-10p-10s	8f-8l-12p-12s	0.659	20	0	0.0		2.9	
	PHH02	*bmos*	3b-3m-3o-3s	4b-2m-3o-3s		6	0	0.0		0.9	
	ZLH05	*afkq*	3a-3f-3k-3q	4a-1f-3k-4q		6	0	0.0		0.9	
						116	0	0.0	1.000	16.6	0.005
E070	PHH06	*abbc*	15a-30b-15c	20a-28b-12c	0.301	14	2	2.3		4.0	
	PHH01	*deei*	3d-6e-3i	4d-5e-3i		6	0	1.0		1.7	
	PHF14	*defi*	12d-12e-12f-12i	17d-9e-8f-14i	0.212	24	0	0.0		3.4	
	PLH04	*befh*	4b-4e-4f-4h	4b-5e-5f-2h		8	0	0.0		1.1	
	ZHH18	*eejk*	8e-4j-4k	8e-5k-3j		8	0	1.3		2.3	
	PLH19	*bdee*	12b-12d-24e	11b-11d-26e	0.846	8	2	1.3		2.3	
	PLH08	*deeh*									
	PLF05	*bbdd*	17b-17d	18b-16d	0.732	12	5	4.0		5.1	
	PHH02	*dffg*	3d-6f-3g	3d-5f-4g		6	0	1.0		1.7	
	ZLH05	*eehl*	6e-3h-3l	6e-2h-4l		6	0	1.0		1.7	
						92	9	12.0	0.197	23.4	0.007
E089	PHH06	*cccc*	52c	52c		14	14	14.0		14.0	
	PHH01	*accc*	3a-9c	3a-9c		6	3	3.0		3.4	
	PHF14	*accc*	12.5a-37.5c	14a-36c	0.624	24	11	12.0		13.7	
	PLH04	*cccc*	16c	16c		8	8	8.0		8.0	
	ZHH18	*bccd*	4b-8c-4d	3b-10c-3d		8	2	1.3		2.3	
	PLH19	*accc*	12a-36c	12a-36c	1.000	8	3	4.0		4.6	
	PLH08	*cccd*	12c-4d	11c-5d		8	3	4.0		4.6	
	PLF05	*aacc*									
	PHH02	*accc*									
	ZLH05	*accc*									
						76	44	46.3	0.131	50.6	0.042

E, expected; O, observed; RCdS, random chromatid segregation; RCeS, random chromosome segregation; ‡, null allele

[a]Including allele frequencies among progenies derived from selfing in crosses A and F.

[b]Chi-squared tests were calculated for larger data sets only, i.e. for parents that were used in multiple crosses (Table 2)

[c]Total number of gametes for which the allelic configuration was determined.

[d]Wilcoxon signed-ranks test for deviation of observed from expected frequency of homozygous gametes.

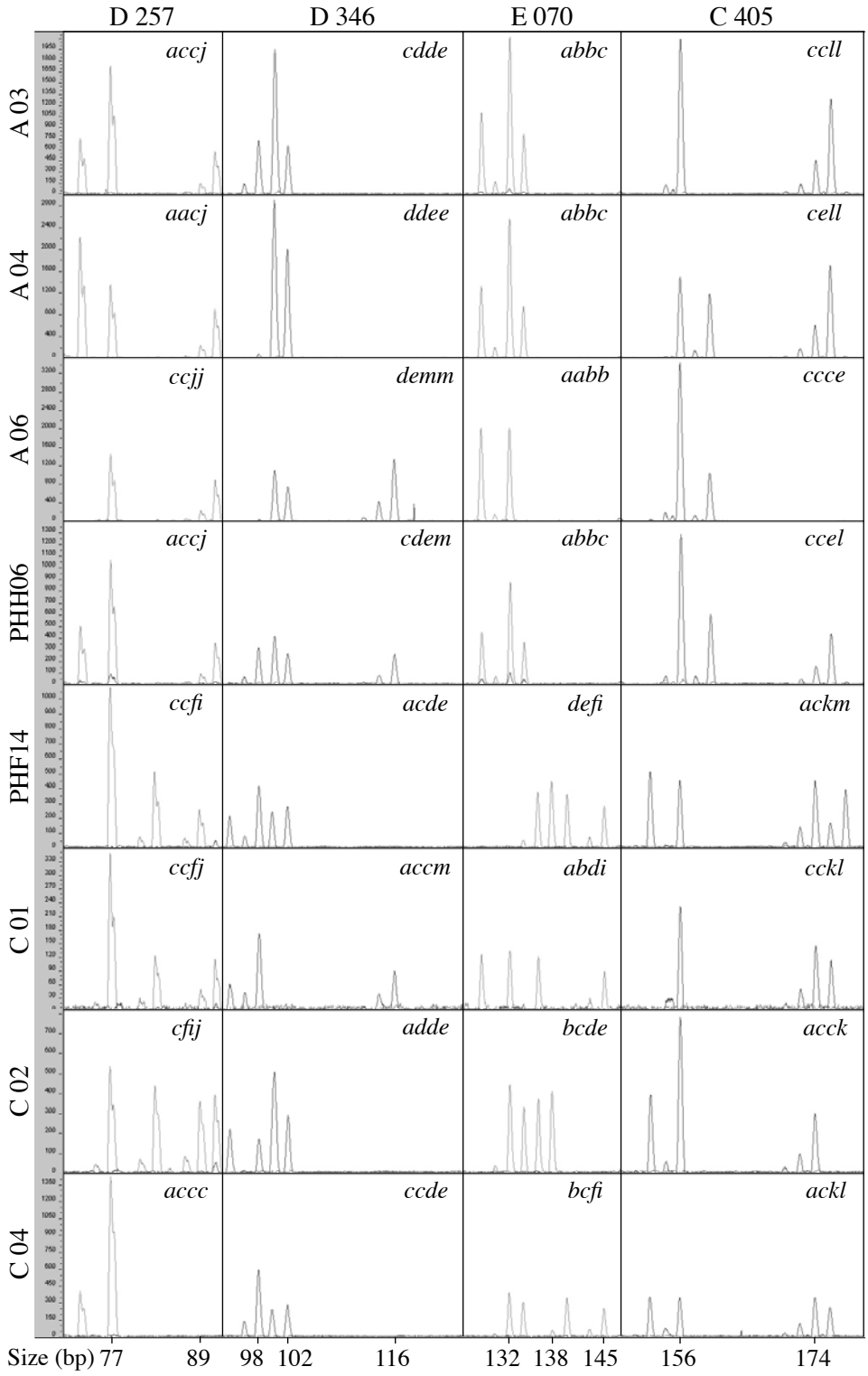

Fig. 1 Electropherograms of four microsatellite loci in eight individuals of tetraploid *Thymus praecox* agg. showing PCR based dosage effects of allele copy number. Progenies A03, A04 and A06 were derived from self-fertilization of hermaphrodite PHH06. Progenies C01, C02 and C04 were derived from controlled crosses of female PHF14 with pollen from PHH06. Amplification products of the four loci C405, D257, D346 and E070 were multiplexed for fragment analysis (peaks of the internal size standard are suppressed in electropherograms).

Supplementary Material I. Sequence alignment of six microsatellite loci and adjacent flanking regions in *Thymus praecox* agg. One sequence per locus, named clone, refers to the one extracted from a partial genomic library and submitted to GeneBank, the other alleles were isolated from individuals from different populations and geographical regions (see materials and methods for abbreviations). The 5′ primers used to generate the sequences are listed. Missing data are indicated by dots. Character substitutions are underlined, indels indicated by dashes. Primer sites used for optimized locus amplification (Table 1) are displayed in bold. Allele sequencies are sorted according to the anticipated size [bp] of corresponding marker fragments. The marker size is underlined for repeat alleles that are identical in state but associated with divergent flanking-sequence variants. Marker size is displayed in bold for different repeat alleles that would not have been discriminated in fragment length analysis due to indels within the marker fragment or due to substitutions within the microsatellite repeat array. For each locus, the number of sequenced alleles, the number of different repeat allele sequences and the number of different marker allele sizes that would have been detected by fragment analysis are listed. The microsatellite allelic variation that would have been missed in fragment analysis was quantified per locus.

Locus C405. Sequences were generated using the primer pairs F1R1, F2R1 and F2R3. Primers: F1 GGTGAAGATGGGATTTGCTAC; F2 AGCATCTCGAAGCGAAAGTC; R1 AATCCATCCCTAGTCACCCC; R3 AACCAAACWWGCRRACTTGTTG. Alleles PLF07 and PLH11 were excluded from the first part of the alignment because sequence electropherograms generated with primer R1 broke down within the microsatellite repeat array. The 24 sequences comprised nine different repeat alleles. Fragment length analysis would have detected 10 different alleles because one allele (GLH08b) would have been erroneously sized due to a deletion in the marker fragment.

Positions 85-180 in GeneBank accession no. AM087136

```
ZLF10a F2   173 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTAAAAGAGG
ZH-05b F1R1 171 .....................................CTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCT--AAAAGAGG
ZHH17a F2   171 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT--AAAAG...
SHF13b F2R1 169 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT----AAAAGAGG
ZHH17b F2   169 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT----AAAAGAGG
ZLF10b F2   169 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT----AAAAGAGG
ZLF25b   R1 169 GGTGAAGATGGGATTTGCTACACCCACCCCGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT----AAAAGAGG
Clone  C405 167 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT------AAAAGAGG
FH-02  F1R1 165 ........................CCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCT--------AAAAGAGG
SHF13a F2R1 165 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCTCTCTCT--------AAAAGAGG
PLH06  F2   161 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCTCTCT------------AAAAGAGG
GLH18  F1R1 157 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCT----------------AAAAGAGG
PHF01  F2   157 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCT----------------AAAAGAGG
ZHH16b F2   157 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCT----------------AAAAGAGG
ZH-05a F1R1 157 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCT----------------AAAAGAGG
PLF08  F2   155 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCTCT------------------AAAAG...
FLF05  F2R1 153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
GLH08a   R1 153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
LLH17  F1R1 153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
PHH12  F2   153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
SLF08    R1 153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
ZHH16a   R1 153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
ZLF25a   R1 153 GGTGAAGATGGGATTTGCTACACCCACCCTGAGAAACTTCCTGGTCTGAACTACCTTCTCTTCTCTCTCTCTCTCTCT--------------------AAAAGAGG
GLH08b   R1 145 ..................ACACCCACCCTGAGAAACTTCCTGGTCTGAACTAC--------CTCTCTCTCTCT--------------------AAAAGAGG
```

Locus C405 continued. No data were available for the second part of the alignment for the alleles ZLF10a, ZHH17a, ZHH17b and PLF08 because sequence electropherograms generated with primer F2 broke down after the microsatellite repeat array.

Positions 181-283 in GeneBank accession no. AM087136

```
ZH-05b F1R1 171 TAGCGAAGTCCGCCAGTCCGCCAGAGAATGCTACAAGTCCGCAAGTTTGCTTGTACGGAAGTCTTATCGTCACATCCACACTTTTTAGGGTTTTTGTTTTCTC
SHF13b F2R1 169 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTCGGAAACTTTTTAGGGTTTTTGTTTTCTC
ZLF10b F2   169 TTGCTTATGCCGCCCGTAATTGAKAGTATTCAACAAG.....................................................................
ZLF25b   R1 169 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTTGGAAACTTTTTAGGGTTTTTGTTTTCTC
Clone  C405 167 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTTGGAAACTTTTTAGGGTTTTTGTTTTCTC
FH-02  F1R1 165 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTTGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTCGGAAACTTTTTAGGGTTTTTGTTTTCTC
SHF13a F2R1 165 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTCGGAAACTTTTTAGGGTTTTTGTTTTCTC
PLH06  F2   161 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTCCGC.....................................................................
GLH18  F1R1 157 TAGCGAAGGCCGCCAGTCCGCCAGAGAATTCAACAAGTCAGCAAGTTTGCTTGTACGGAAGTCTTATCGTCACATCCACACTTTTTAGGGTTTTTGTTTTCTC
PHF01  F2   157 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTCCGCAAGTTTGGTT.....................................................
ZHH16b F2   157 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAG.....................................................................
ZH-05a F1R1 157 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTTGCTTGTTTGGTTTTACTAAAGTCTTATCCTCAC--------TTTTTAGGGTTTTTGTTTTCTC
FLF05  F2R1 153 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTTGGAAACTTTTTAGGGTTTTTGTTTTCTC
GLH08a   R1 153 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTTGCTTGTTTGGTTTTACTAAAGTCTTATCCTCAC--------TTTTTAGGGTTTTTGTTTTCTC
LLH17  F1R1 153 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTTGGAAACTTTTTAGGGTTTTTGTTTTCTC
PHH12  F2   153 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTCCGCAAGTTTGGTT.....................................................
SLF08    R1 153 TWGCKWAKKCCGCCMGTMMKYSAKAGWATTCWACAAGTYWGCWWGTTTGSTTGTACKRAAGTCTTATCSTCACATSSAMACTTTTTAGGGTTTTTGTTTTCTC
ZHH16a   R1 153 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTTGCTTGTTTGGTTTTACTAAAGTCTTATCCTCAC--------TTTTTAGGGTTTTTGTTTTCTC
ZLF25a   R1 153 TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTTGCTTGTTTGGTTTTACTAAAGTCTTATCCTCAC--------TTTTTAGGGTTTTTGTTTTCTC
GLH08b   R1 145 TAGCGAAGTCCGCCAGTCCGCCAGAGAATGCTACAAGTCAGCAAGTTTGSTTGTACGGAAGTCTTATCGTCACWYSSAMACTTTTTAGGGTTTTTGTTTTCTC
PLF07    R1     TTGCGAAGTCCGCCAGTCCGCCAGAGAATGCAACAAGTCCGCAAGTTTGCTTGTACGGAAGTCTTATCGTCACATCCACACTTTTTAGGGTTTTTGTTTTCTC
PLH11    R1     TTGCTTATGCCGCCCGTAATTGATAGTATTCAACAAGTTCGCTTGTTTGGTTTTACTAAAGTCTTATCCTCACTCGGAAACTTTTTAGGGTTTTTGTTTTCTC
```

Locus D257. Sequences were generated using the primer pairs F1R1, F1R2 and F3R3. Primers: F1 AGGCTTCATTCTATACCTCGTG; F3 CTCCRAACMCCCAAACTATTCC; R1 TTCTCTGTTTCCGATCCATGGC; R2 CGATCCATGGCTTGACATGTGC; R3 AAGCATAGTTCTGAAGCACC. No data were available for the first part of the alignment for the alleles sequenced with primer R3 because sequence electropherograms broke down after the microsatellite repeat array.

Positions 132-231 in GeneBank accession no. AM087138

```
SLF08   F1    115 TTCACCTGCATTAAATTAATCTGTCTAGGAAGTTTGTTGAAAGGGAACGCAACTAAAAATTTCTAATCTTCAGATCTCCTAAACAACATTTTTTACATTCGCTATCCATGAAAAA-T
SHF13a  F1     91 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACGACATTTTTTCCATTCGCTATCCATGATTAA-T
SHF13b  F1     91 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACGACATTTTTTCCATTCGCTATCCATGATTAAAT
ZHH16b  F1R1   91 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAG-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGATGAAAT
ZLF25b  F1     89 TTGGGACGGA-GCGAGTAGTATACGTAGTAA--A--------------CAAA-------TTTCTAATCTTCAGATTTCCTTAACAACATTTTTTACATTCGCTATCCATGATTAA-T
Clone   D257   87 TTGGGACGGA-G------------TTAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTATAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGATTAA-T
PHF12b  F1R1   85 TTGGGACGGA-GCGAGTAGTATACGTAGTAA--ACGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGAAAAA-T
ZH-05   F1R1   81 TTGGGACGGA-GCGAGTAGTATACGTAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACGACATTTTTTCCATTCGCTATCCATGAAAAAAT
PLF07   F1R1   79 TTGGGACGGA-GCGAGTGGTATACGTAGTAA--A--------------CAAA-------TTTCTAATCTTCAGATTTCCTTAACAACATTTTTTACATTCGCTATCCATGATTAA-T
PHF12a  F1     77 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGATAAA-T
PHF11   F1     75 TTGGGACAGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCACAAACTAAAAATTTCTAATCTTCAGATTTCCTAAACAACATTTTTTACATTCGCTATCCATGAAAAAAT
FH-01   F1R1   75 TTGGGACGGA-GCGAGTAGTATACGTAGTAA--A--------------CAAA-------TTTCTAATCTTCAGATTTCCTTAACAACATTTTTTACATTCGCTATCCATGATTAA-T
FLF05b  F1     75 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGATTAAAT
LLH17   F1     75 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGATTAAAT
PLH11   F1     75 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTCCATTCGCTATCCATGATTAAAT
ZL-09b  F1R1   73 TTGGGACGGA-GCGAGTAGTATACATAGTAA--AGGCTGGAA-ACACCCAAACTAAAAATTTCTAATCTTCAGATTTCCTTAACAACATTTTTTACATTCGCTATCCATGAAAAAAT
ZLF25a  F1     73 TTGGGACGGA-GCGAGTAGTATACGTAGTAA--A--------------CAAA-------TTTCTAATCTTCAGATTTCCTTAACAACATTTTTTACATTCGCTATCCATGATTAA-T
ZL-09a  F1R1   71 TTGGGACGGA-G------------TTAGTAA--AGGCTGGAA-ACCCCCAAACTAAAAATTTATAATCTTCAGATTTCCTT---AACATTTTTTCCATTCGCTATCCATGATTAA-T
```

Locus D257 continued. The 29 sequences comprised 23 different repeat alleles. Fragment length analysis would have detected 12 different alleles, 48% of the microsatellite allelic variation would have been missed.

Positions 232-320 in GeneBank accession no. AM087138

```
SLF08   F1    115 TCTCCAAACCCCCAAACTATTCCAGAGAGAGAGAGAGGGAGAGAGAGGGAGAGAGAGAGAGAGAGAGAGAGAGAGTTATCAAG.............................
SHF13a  F1     91 TCTCCAAACCCCCAAACTATTCCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG----------------------TTATCAAGAATCTTGCACATGTCAAGCC........
SHF13b  F1     91 TCTCCAAACCCCCAAACTATTCCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG----------------------TTATCAAGAATCTTGCACATGTCAAGCC........
ZHH16b  F1R1   91 TCTCCAAACCCCCAAACTATTCCACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG----------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
FLF05a    R3   91 ...............CTATTCCACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG----------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
ZLF25b  F1     89 TCTCCAAACCCCCAAACTATTCCACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG------------------------TTATCAAGAATCTTGCACATGTCGAGCCATGGATCG
Clone   D257   87 TCTCCGAACACCCAAACTATTCCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
LHF18a    R3   87 ...............CTATTCCACAGAGAGAGAGAGAGAGAGAGAGAGAGAG--------------------------TTATCAAGAATCTTGCACATTTCAAGCCATGGATCG
SLF04     R3   85 ...............CTATTCCAGAGAGAGAGAGAGAGAGAGAGAGAGAG--------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PHF01b    R3   85 ...............CTATTCCACAGAGAGAGAGAGAGAGAGAGAGAGAG--------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PHF12b  F1R1   85 TCTCCAAACCCCCAAACTATTCCACAGAGAGAGAGAGAGAGAGAGAGAGAG--------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PLH06     R3   83 ...............CTATTCCAKAKAGAGAGAGAGAGGGAGAGAGAG--------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
ZHH16a    R3   81 ...............CTATTCCAGAGAGAGAGAGAGAGAGAGAGAGAG--------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
ZH-05   F1R1   81 TCTCCAAACCCCCAAACTATTCCATAGAGAGAGAGAGAGAGAGAGAGAG--------------------------TTATCAAGAATCTTGCAC.................
PLF07   F1R1   79 TCTCCAAACCCCCAAACTATTCCACAGAGAGAGAGAGAGAGAGAG------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
LHF18b    R3   77 ...............CTATTCCAGAGAGAGAGAGAGAGAGAG------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
ZHH17     R3   77 ...............CTATTCCAGAGAGAGAGAGAGAGAGAG------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PHF12a  F1     77 TCTCCAAACCCCCAAACTATTCCATATAGAGAGAGAGAGAGAG------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PLF08     R3   77 ...............CTATTCCAKAKAGAGAGAGAGAGAGAG------------------------------TTATCAAGAATCTTGCACATGTCAAGCCGTGGATCG
SHF13c    R3   77 ...............CTATTCCACAGAGAGAGAGAGAGAGAG------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PHF11   F1     75 TCTCCAAACCCCCAAACTATTCCAGAGAGAGAGAGAGAGAG--------------------------------TTATCAA.............................
FH-01   F1R1   75 TCTCCAAACCCCCAAACTATTCCACAGAGAGAGAGAGAGAG--------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
FLF05b  F1     75 TCTCCAAACCCCCAAACTATTCCATATATAGAGAGAGAGAG--------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
LLH17   F1     75 TCTCCAAACCCCCAAACTATTCCATAGAAAGAGAGAGAGAG--------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
PLH11   F1     75 TCTCCAAACCCCCAAACTATTCCATAGAGAGAGAGAGAGAG--------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
ZL-09b  F1R1   73 TCTCCAAACCCCCAAACTATTCCATAGAGAGAGAGAGAG------------------------------------TTATGAAGAATCTTGCACATGTCAAGCCATGGATCG
ZLF25a  F1     73 TCTCCAAACCCCCAAACTATTCCATAGAGAGAGAGAGAG------------------------------------TTATGAAGAATCTTGCACATGTCAAGCCATGGATCG
PHF01a    R3   73 ...............CTATTCCAGAGAGAGAGAGAGAG------------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
ZL-09a  F1R1   71 TCTCCAAAGCCCCAAACTATTCCAGAGAGAGAGAGAG------------------------------------TTATCAAGAATCTTGCACATGTCAAGCCATGGATCG
```

Locus D346. Sequences were generated using the primer pairs F2R1 and F3R1. Primers: F2 GAAAGGAGAAGTTAGGGGAAAG; F3 TGATTAATCAGTATAACACCATGG; R1 CTGTGTCAGTCTGCAAAAG. The 26 sequences comprised 10 different repeat alleles. Fragment length analysis would have detected nine different alleles, 10% of the microsatellite allelic variation would have been missed.

Positions 320-428 in GeneBank accession no. AM087139

```
PHF12c F2R1 114 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCGTGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGGTTGGTATGTGTTTTTGAGAG
PLH11b F3   114 ..............................................................AGAGATCTAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGGTTGGTRTGTGTTTKWGAGAG
PHF12d F2       ATTACTCCTTTAGTTTAACATCATCCCCATAATAGCARCACCATGATTAATCAGTATAACACCATGGTCAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAGAG.........................
FH-03  F2   108 ATTACTCCTTTAGTTTAACATCATCCCCATAATAGCARCACCATGATTAATCAGTATAACACCATGGTCAGCGATCTAGAGAGAGAGAGAGAGAGAGAGAGAG------GGGTTGGTGTGTGTTT.......
PLF08  F2   108 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCGTGATTAATCAGTATAACACCATGGCCAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAGAG------GGGTTGGTGTGTGTTT.......
PLH04a F2   108 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCGTGATTAATCAGTATAACACCATGGCCAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAGAG------GGGTTGGTATGTGTTTTTGAGAG
SHF13b F3   108 ..............................................................ATCTAGAGAGAGAGAGAGAGAGAGAGAG------AGGTTGGTATGTGTKTKTGAGAG
PHF01  F3   106 ..............................................................AGAGATCTAGAGAGAGAGAGAGAGAGAGAG--------AGGTTGGTATGTGTTTTTGAGAG
PHF12b F2R1 106 ATTACTCCTTTAGTTTAACATCATCCCCATAATAGCARCACCGTGATTAATCAGTATAACACCATGGCCAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAG--------AGGTTGGTATGTGTTTTTGAGAG
PHH17  F2   106 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCGTGATTAATCAGTATAACACCATGGCCAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAG--------AGGTTGGTGTGTGTGTTTGAGAG
PLH11a F3   106 ..............................................................AGAGATCTAGAGAGAGAGAGAGAGAGAGAG--------AGGTTGGTATGTGTTTTTGAGAG
ZHH17b F3   106 ..............................................................AGAGATCTAGAGAGAGAGAGAGAGAGAGAG--------AGGTTGGTATGTGTGTTTGAGAG
Clone  D346 104 ATTA------------ACATAAACCCCATAAAAGCAACACCATGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAGAGAGAGAGAG----------AGGTTGGTATGTGTTTTGAGAG
PLH04c F2R1 104 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCATGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAGAGAGAGAGAGAG----------AGGTTGGTATGTGTTTTTGAGAG
SLF08b F3   104 ..............................................................AGAGATCTAGAGAGAGAGAGAGAGAGAGAG----------AGCTTGGTATGTGTGTTTGAGAG
ZLF10  F3   104 ..............................................................GAGATCTAGAGAGAGAGAGAGAGAGAGAG----------AGGTTGGTATGTGTKTKTGAGAG
PHF12a F2R1 102 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACTATGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAGAGAGAGAG----------AGGTTGGTATGTGTTTTGAGAG
ZL-09a F2   102 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCGTGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAGAGAGAGAG----------GGG.................
PLH06  F3   102 ..............................................................AGAGATCTAGAGAGAGAGTGAGGGAGAG----------AGCTTGGTATGTGTTTTTGAGAG
LLF02  F3   102 ..............................................................AGAGATCTAGAGAGAGAGTGAGGGAGAG----------AGCTTGGTATGTGTTTTTGAGAG
LLH14  F3   100 ..............................................................AGAGATCTAGAGAGAGAGAGAGAGAG----------AGCTTGGTATGTGTTTTTGAGAG
SLF08a F3   100 ..............................................................AGAGATCTAGAGAGAGAGAAGAAGAG----------AGCTTGGTATGTGTTTTTGAGAG
SHF13a F3    98 ..............................................................AGAGATCTAGAGAGAGAGAGAGAG----------AGCTTGGTATGTGTTTTTGAGAG
ZHH17a F3    98 ..............................................................AGAGATCTAGAGAGAGAGAGAGAG----------AGSTTGGTATGTGTTTTTGAGAG
GLH10  F2R1  94 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACCGTGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAG----------AGCTTGGTATGTGTTTTTGAGAG
PLH04b F2R1  94 ATTACTCCTTTAGTTTAGCATAAACCCCATAAAAGCAACACCATGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAGAG----------AGCTTGGTATGTGTTTTTGAGAG
ZL-09b F2R1  92 ATTACTCCTTTAGTTTAACATAAACCCCATAAAAGCAACACTATGATTAATCAGTATAACACCATGGACAGAGATCTAGAGAGAGAG----------GGGTTGGTATGTGTTTTTGAGAG
```

Locus D347. Sequences were generated using the primer pair F1R2. Primers: F1 TTTAGTGATAAAGTAACAACACC; R2 GTGCCTCCTTCTATTCATCAC. The reverse primer site (positions 252-272) of the marker fragment is not shown. The 18 sequences comprised 17 alleles. Fragment length analysis would have detected 14 different alleles, 17.6% of the allelic variation would have been missed.

Positions 143-251 in GeneBank accession no. AM087140

```
PHF13b F1R2 142 TACACACACAGTGKAGGTGTAAGTGTGTGTTGATTTGATT--------AGAACAGAAGAGTTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGG--AGAAGGAACCTTGAGAGAGAGGGATGAGCACAGTG
PLH04c F1R2 140 CACACACACAGTGKAGGTGCAAGTGTGTGTTAATTTGGTT--------AGAACAGGGGAGA-GAGAGAGAGAGAGAGAGAGAGAGAGAGGGAAGAAGGAACCTTGAGAGAGAGGGA---GCACAGTG
ZLH05d F1R2 136 CACACACAGAGTGGAGGTGCAAGTGTGTGTTAATTTGATT--------AGAACAGAAGAGAGGGGAGAGAGAGAGAGAGAGAGAGA--------GAAGAAGAAACCTTGAGAGAGAGGGATGAGCACAGTG
PHH09b F1R2 134 CACACACACAGTGGAGGTGAAAGTGTGTGTTAATTTGATTAGAAATTTAGAACAGAAGAGAGGAGAGAGAGAGAGAGAGA--------------GAAGGAACCTTGAGAGAGAGGGA---GAACAGTG
GLH08b F1R2 131 CACACACACAGTGGAGGTGAAAGTGTGCGTTAATTTGATTAGAAATTTAGAACAGAAGAGAGGAGAGAGAGAGAGA--------------------GAAGGAACCTTGAGAGAGAGGGATGAGCACAGTG
PHH09a F1R2 130 CACACACACAGTGGAGGTGCAAGTGTGTGTTAATTTGGTT--------AGAACAGGGGGGA-GAGAGAGAGAGAGAGAGAGA----------GGGAAGAAGGAACCTTGAGAGAGAGGGA---GCACAGTG
Clone  D347 130 CACACACACAGTGGAGGTGCAAGTGTGTGTTAATTTGGTT--------AGAACAGGGGGGA-GAGAGAGAGAGAGAGAGAGA----------GGGAAGAAGGAACCTTGAGAGAGAGGGA---GCACAGTG
PHF13a F1R2 128 CACACACACAGTGKAGGTGCAAGTGTGTGTTAATTTGGTT--------AGAACAGGGGGGA-GAGAGAGAGAGAGAGAGAGA----------GGGAAGAGGGAACCTTGAGAGAGAGGGA---GCACAGTG
FH-01b F1R2 128 CACACACACAGCGGAGGTGAAAGTGTGTGTTAATTTGATTAGAAATTTAGAACAGAAGAGAGGAGAGAGAGAGAGA--------------------GAAGGAACCTTGAGAGAGAGGGA---GAACAGTG
ZHF11  F1R2 128 TACACACACAGTGGAGGTGTAAATGTGTGTTGATTTGATT--------AGAACAGAAGAGTTGAGAGAGAGAGAGAGA----------------GAAGAAGGAACCTTGAGAGAGAGGGATGAGCACAGTG
ZLH05c F1R2 127 CACACACACAGTGGAGGTGCAAGTGTGTGTTAATTTGGTT--------AGAACAGGGGGGA-GAGAGAGAGAGAGA----------------GGGAAGAAGGAACCTTGAGAGAGAGGG.............
GLF09  F1R2 123 CACACACACAGTGGAGGTG-AAGTGTGTGTTAATTTGATT--------AGAACAGAAGAGAGGAGAGAGAGAGAGAGAGAGAGA----------CCTTGAGAGAGAGGAATGAGCACAGTG
FH-01a F1R2 122 TACACACACAGTGGAGGTGTAAGTGTGTGTTAATTTGATT--------AGAACAGAAGAGAGGGGAGAGAGAGAGAGAGAGA----------CCTTGAGAGAGAGGGATGAGCACAGTG
GLH08a F1R2 122 CACACACACAGTGGAGGTGCAAGTGTGTGTAAATTTGATT--------AGAACAGAAGAGAGGAGAGAGAGAGAGAGA----------GAAGGAACCTTGAGAGAGAGGGA---GCACAGTG
PLH04b F1R2 121 CACACACACAGTGGAGGTGCAAGTGTGTGTTAATTTGATT--------AGAACAGAAGA--GGAGAGAGAGAGAGA----------GAAGAAGGAACCTTGAGAGAGAGGGA---GCACAGTG
ZLH05b F1R2 118 CACACACACAGTGAAGGTGCAAGTGTGTGTTAATTTGATT----------AGAGAGAGAGAGAGAGAGAGAGAGAGA----------GAACCTTGAGAGAGAGGGATGAGCACAGTG
PLH04a F1R2 115 CACACACACAGTGGAGGTG-AAGTGTGTGTTAATTTGATT--------AGAACAGAAGAGAGGAGAGAGAGAGAGA----------CCTTGAGAGAGAGGGATGAGCACAGTG
ZLH05a F1R2 106 CACACACACAGTGGAGGTG-AAGTGTGTGTTAATTTGATT----------AGAAGAGAGGAGAGAGAGA----------CCTTGAGAGAGAGGGATGAGCACAGTG
```

Locus E070. Sequences were generated using the primer pairs F2R1, F4R1 and F1R6. Primers: F1 GGTGTAGTACCTGTAGGGATTG; F2 CTCCCATGTTGCAGTTACTGC; F4 CCCATGTTGYAGTTACTGCG; R1 CTCGAGCTCAACTCAACTCAG; R6 GCCATTTTCATYYMTMTCTCC. The forward primer site (positions 271-290) of the marker fragment is not shown. The 27 sequences comprised 11 different repeat alleles. Fragment length analysis would have detected 10 different alleles; 9% of the microsatellite allelic variation would have been missed and, in addition, two alleles would have been confused with others due to deletions in the marker fragment.

Positions 301-414 in GeneBank accession no. AM087141

```
ZLF25b F4R1 155 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGGAGAGAGAGAGAGGAGATAGAGATGAAAATGGCGGAG
FLF05a F4R1 153 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGA--GGAGAGAGAGAGAGAGGAGATAGAGATGAAAATGGCGGAG
LLH14a F4R1 153 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGA--GGAGAGAGAGAGAGAGGAGATAGAGATGAAAATGGCGGAG
ZL-O9  F2R1 152 GTCTACTTTTGTTGGTTAGGAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTGTAGAGAGAGAGAGAGAGAGAGAGA-GAGAGAGAGAGA--GGAGATAGAAATGAAAATGGCGGAG
FH-01  F2R1 150 GTTTAGTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA-GAGAGAGAGA----GGAGATATAAATGAAAATGGCGGAG
ZLF03b F1   144 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA-GAGA----------GGAGATATAAATGAGAATGGC....
GLF09  F2R1 142 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA-GA----------GGAGATATAAATGAAAATGGCGGAG
ZLF03c F4   140 GTTTACTTTTGTTGGTTAGGAGAGAATGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCG----GAGAGAGAGAGAGAGAGAGA-GAGA----------GGAGATATATATGAAAATGGCGGAG
GLH08a F2R1 140 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATAGAAATGAAAATGGCGGAG
PHF13  F2R1 140 GTTTAGTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTCAAGAGAGAGAGAGAGAGAGAGA----------GGAGAGAGGAATGAAAATGGCGGAG
Clone  E070 140 GTTTAGTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAAAGAGAGAGA----------GGAGATAGAAATGAAAATGGCGGAG
PLH06  F1   140 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAAAGAGAGAGA----------GGAGATATAAATGAAAATGGC....
LLF17  F1   138 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATATAAATGAAAATGG.....
LLH14b F4R1 138 GTTTAGTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATAGAGATGAAAATGGCGGAG
PHF01    R1 138 GTTTACTTTTGTTGTGTACTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTCAAGAGAGAGAGAGAGAGAGAGA----------GGAGATATAAATGAAAATGGCGGAG
ZLF03a F1   138 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATATAAATGAAAATGGC....
ZLF10  F2R1 138 GTTTAGTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATATAAATGAAAATGGCGGAG
ZLF25a   R1 138 GTTTAGTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATAGAAATGAAAATGGCGGAG
PLH08  F2R1 138 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGCCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATAGAAATGAAAATGGCGGAG
SLF08a F4R1 138 GTCTACTTTTGTTGGATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTGCTTCGTTAAGAGAGAGAGAGAGAGAGAGA----------GGAGATATAAATGAAAATGGCGGAG
GLH08b F2R1 136 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTC--GAGAGAGAGAGAGAGAGAGA----------GGAGATAGAAATGAAAATGGCGGAG
ZHF08  F4R1 136 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTCAAGAGAGAGAGAGAGAGAGA----------GGAGATATAAATGAAAATGGCGGAG
ZHH16  F1   136 GTYTACTTTTGTTGKATRSKAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTYAAGAGAGAGAGAGAGAGAGA----------GGAGATATATATGAAAATGGC....
FLF05b F1   136 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGA----------GGAGATATATATGAAARTGGC....
FLF13  F1   136 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGAGAGAGAGA----------GGAGATATATATGAAAATGGC....
PHH10  F1   130 GTTTACTTTTGTTGTATGCTAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTTGTTCGTTAAGAGAGAGAGA----------GGAGATATAAATGAAAATGGC....
SLF08b F1   130 GTCTACTTTTGTTGGTTAGGAGAGAAGGCAGAAAGCGGTTAGTCTCTTCTTCCTCCCTTGCTTCGTTTAGAGAGAGAGA----------GGAGATATAAATGAAAATG......
```

Locus E089. Sequences were generated using the primer pairs F1R1, F1R2 and F1R4. Primers: F1 GCAAGAGAAACTAAACTCCCTC; R1 GAGGGAGTTTAGTTTCTCTTGC; R2 CATTTGTAGCAGCTGTACAGCC; R4 GAAGAAGTAAACCTGAAATCC. The reverse primer site (positions 250-270) of the marker fragment is not shown. The 26 sequences comprised four different alleles, which would also have been found in fragment analysis.

Positions 110-209 in GeneBank accession no. AM087142

```
LLH14b   R4 137 GATCAAACTTCTCATAAACAAA-G---------AAG**AAGAGAAGAGGAGAAGAA**GAAGAAGAAGAAATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
Clone  E089 134 GATCAAACTTCTCATAAACTAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAAGAA---ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
PHF12a F1R1 134 GATCAAACTTCTCATAAACTAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAAGAA---ATGGGGGATTTGATCATGG..........................
PLF07  F1R2 134 GATCAAACTTCTCATAAACTAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAAGAA---ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
PHF03b   R4 134 GTTCATCCTTCTCATCATCTACA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAAGAA---ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
PHF12b F1R1 134 GTTCATCCTTCTCATCATCTACT----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAAGAA---ATGGGGGATTTGATCATGGGTCCG.....................
GLH18  F1R1 131 GATCAAACTTCTCATAAACAAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
ZL-09b F1   131 GATCAAACTTCTCATAAACAAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
PHF11  F1R1 131 GATCAAACTTCTCATAAACTAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGGTCCG.....................
LHF18    R4 131 GATCAAACTTCTCATAAACTAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
GLF02a F1R2 131 GATCAAACTTCTCATAAACTAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTAC.....
FH-04    R2 131 GATCAAACTTCTCATAAACAAA----------GAG**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGG.........................
GLF02b   R2 131 GATCAAACTTCTCATAAACTA---------------**AAGAGAAGAGAAGAAGAA**GAAGAA------ATGGGGGATTTGATAATGGGTCCGA.....................
GLH02a F1   131 GATCAAACTTCTCCTAAACTA---------------**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
ZL-09a F1   131 GATCAAACTTCTCATAAACAA---------------**AAGAGAAGAGGAGAAGAA**GAAGAA------ATGGGGGATTTGATAATGGGTCCGAGGCTGTACAGCTGCTACAAATG
GLH02b   R2 125 GATCAAACTTCTCATAAACAAA-GAGAAGAGAAGAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGA.....................
ZH-05  F1   125 GATCAAACTTCTCATAAACAAA-GAGAAGAGAAGAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
LLF03    R4 125 GATCAAACTTCTCATAAACAAAA----------GAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
LLH14a   R4 125 GATCAAAGTTCTCATAAACAAAA----------GAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTC.....................
ZHH16    R4 125 GATCAAACTTCTCATAAACAAAA----------GAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
PHF03a   R4 125 GATCAAACTTCTCATAAACAAAA----------AAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTG.................
ZHH17    R4 125 GATCAAACTTCTCATAAACAAAA----------AAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
ZL-09c F1R2 125 GATCAAACTTCTCATAAACAAAA----------AAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGT.....................
ZLF03    R4 125 GATCAAACTTCTCATAAACAAAA----------AAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
SHF13    R4 125 GATCAAACTTCTCATAAACAAA----------GAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
SLF14    R4 125 GMTCAMWCTTCTCATAAACAAA----------GAG**AAGAGAAGAGAAGAAGAA**------------ATGGGGGATTTGATCATGGGTCCGAGGCTGTACAGCTGCTACAAATG
```

Supplementary Material II. Estimates of allelic dosage and assignment of allelic configuration in 10 controlled crossing families of tetraploid *Thymus praecox* agg. Progenies are listed below one parent (see Table 2 for the complete crossing design). Electropherogram peak heights were corrected for first and second order stutter products in loci C405, D257, D346 and D347. Alleles with low relative amplification intensity are framed with a dashed line, overamplified alleles with a bold line. Wherever possible, genotypes of parental ovules and pollen were deduced from parental allelic configurations.

Locus C405

	Allele peak height													Genotype		
Individual	152-a	154-b	156-c	158-d	160-e	162-f	164-g	166-h	168-i	170-j	174-k	176-l	178-m	Individual	Ovule	Pollen
PHH06			1307		612							615		*ccel*		
A01			407		359							810		*cell*		
A02			602		620							1053		*cell*		
A03			2276									1989		*ccll*		
A04			1509		1374							2601		*cell*		
A05			3179		1546							1515		*ccel*		
A06			3471		1053									*ccce*		
A07			2174									1930		*ccll*		
A08			5075									3806		*ccll*		
PHH01	1096		1107	1027	896									*acde*		
B01			657	316	291									*ccde*	*cc/ce*	*de/cd*
B02	1310				2324							1086		*aeel*	*el*	*ae*
B03			2364	2374	2368							2110		*cdel*	*cl/el*	*de/cd*
B04			3347	1426								1418		*ccdl*	*cl/el*	*cd*
B05			2965	1263	1047									*ccde*	*cc/ce*	*de/cd*
B06	1690		3358		1524									*acce*	*cc/ce*	*ae/ac*
PHF14	527		461								611		580	*ackm*		
C01			278								149	157		*cckl*	*ck*	*cl*
C02	400		865								415			*acck*	*ak*	*cc*
C03	338		831									383		*accl*	*ac*	*cl*
C04	367		360								362	361		*ackl*	*ak*	*cl*
C05	703		664		672							668		*acel*	*ac*	*el*
C06	651		619								666	634		*ackl*	*ak*	*cl*
C07			2631								1136	1169		*cckl*	*ck*	*cl*
C08			2054		1912							1809	1691	*celm*	*cm*	*el*
PLH04	2193		853											*aaac*		
D01	540		383										233	*aacm*		
D02	617												197	*aaam*	*am*	*aa*
D03	346										117			*aaak*	*ak*	*aa*
D04	1199		674										631	*aacm*		
D05	1192		660										644	*aacm*		
D06	2298		1163										1117	*aacm*		
D07	3342										1005			*aaak*	*ak*	*aa*
D08	2242												670	*aaam*	*am*	*aa*
ZHH18									1621	1584				*iijj*		
E01	773								792	785			727	*aijm*	*am*	*ij*
E02	466		537						373	403				*acij*	*ac*	*ij*
E03	1158								1140	1192			1111	*aijm*	*am*	*ij*
E04										2050	1008		925	*jjkm*	*km*	*jj*
E05	913		961						855	796				*acij*	*ac*	*ij*
E06	1330								1270	1273	1048			*aijk*	*ak*	*ij*
E07									2729		1277		1256	*iikm*	*km*	*ii*
E08									1250		597		523	*iikm*	*km*	*ii*
PLH19	5549			2728	2749									*aade*		
F01	5610			2638	2646									*aade*		
F02	3633			1337										*aaad*		
F03	4322				1319									*aaae*		
F04	2504			1204	1314									*aade*		
F05	2149			1093	932									*aade*		
F06	1522			1472										*aadd*		
F07	1838			922	933									*aade*		
F08	1609			2831	1215									*adde*		
PLH08	2987		1289							1211				*aacj*		
G01	2973		1337		1280									*aace*	*ae*	*ac*
G02	162			132	120					99				*adej*	*de*	*aj*
G03	2228				705									*aaae*	*ae*	*aa*
G04	3640			1729						1575				*aadj*	*ad*	*aj*
G05	2471		1156		1090									*aace*	*ae*	*ac*
G06	2860				1319					1303				*aaej*	*ae*	*aj*
G07	3023			1447	1345									*aade*	*de*	*aa*
G08	3761				983									*aaae*	*ae*	*aa*
PLF05			1748		1672	1751			1402					*cefi*		
H01	4982				4798	4760			4373					*aefi*	*fi*	*ae*
H02	2956		2722		2779	2778								*acef*	*cf*	*ae*
H03	999				944	978			748					*aefi*	*fi*	*ae*
H04			1528	1510	2126									*cdee*	*ce*	*de*
H05			1490	1281	1137				1019					*cdei*	*ci*	*de*
H06	1296			1351	1135				1098					*adei*	*ei*	*ad*
H07	2267				1102	1109								*aaef*	*ef*	*aa*
H08	517				949	437								*aeef*	*ef*	*ae*
PHH02		1198	1124				1111			924				*bcgj*		
I01		2074	3844			2027								*bccf*	*cf*	*bc*
I02			2822			1418				1327				*ccfj*	*cf*	*cj*
I03			725				575		515	528				*cgij*	*ci*	*gj*
I04			3517		1585		1667							*cceg*	*ce*	*cg*
I05		1366				1311			1091	1156				*bfij*	*fi*	*bj*
I06			1199		1119	1175				1005				*cefj*	*ef*	*cj*
ZLH05	534	518						507		507				*abhj*		
K01	3311				3137			2934	2667					*aehi*	*ei*	*ah*
K02	2315		2190		2178					2085				*acej*	*ce*	*aj*
K03		702	739					637	579					*bchi*	*ci*	*bh*
K04		891			867	921				870				*befj*	*ef*	*bj*
K05	1216	1008			1066	1000								*abef*	*ef*	*ab*
K06	1176	1035			1018	898								*abef*	*ef*	*ab*

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	Allele peak height													Genotype		
Individual	073-a	075-b	077-c	079-d	081-e	083-f	085-g	087-h	089-i	091-j	097-k	101-l	103-m	107-n	Individual	Ovule	Pollen
PHH06	523		1082							471					*accj*		
A01	430		1067							470					*accj*		
A02	600		1961												*accc*		
A03	767		1781							771					*accj*		
A04	2270		1398							1172					*aacj*		
A05	898		2747												*accc*		
A06			1493							1235					*ccjj*		
A07	731		2456												*accc*		
A08	1704		1900												*aacc*		
PHH01			697				238			213					*ccgj*		
B01			503				344			755					*cgjj*	*cj*	*gj*
B02			1820				431								*cccg*	*cc*	*cg*
B03			1881				664			708					*ccgj*	*cc/cj*	*gj/cg*
B04	825		1605				659								*accg*	*ac*	*cg*
B05	593		1175							441					*accj*	*ac*	*cj*
B06			1842							497					*cccj*	*cc/cj*	*cj/cc*
PHF14			1093			618			352						*ccfi*		
C01			355			165				180					*ccfj*	*cf*	*cj*
C02			602			539			471	501					*cfij*	*fi*	*cj*
C03	372		566						446	448					*acij*	*ci*	*aj*
C04	472		1436												*accc*	*cc*	*ac*
C05	662		1555						594						*acci*	*ci*	*ac*
C06			1357			614				558					*ccfj*	*cf*	*cj*
C07			1960							635					*cccj*	*cc*	*cj*
C08	870		945			915			693						*acfi*	*fi*	*ac*
PLH04	718		708								343	374			*ackl*		
D01			770						500		449	410			*cikl*	*ci*	*kl*
D02			302						179		139	188			*cikl*	*ci*	*kl*
D03	137		154			149						118			*acfl*	*cf*	*al*
D04	483		1041									338			*accl*	*cc*	*al*
D05						532			347		339	346			*fikl*	*fi*	*kl*
D06			1607						579		489				*ccik*	*ci*	*ck*
D07	671		1248						545						*acci*	*ci*	*ac*
D08	547		980			534									*accf*	*cf*	*ac*
ZHH18					883	551			368						*eefi*		
E01			708		606	505			556						*cefi*	*ci*	*ef*
E02			576		440				887						*ceii*	*ci*	*ei*
E03			1557		1381										*ccee*	*cc*	*ee*
E04					193	142			311						*efii*	*fi*	*ei*
E05			929		934	1572			636						*cefi*	*c(f/i)*	*e(f/i)*
E06			804			1464			1336						*cfii*	*ci*	*fi*
E07			1024		997				1577						*ceii*	*ci*	*ei*
E08			1267		748	977									*ccef*	*cc*	*ef*
PLH19	1193		2172			1094									*accf*		
F01	1957		1892												*aacc*		
F02	1120		3186												*accc*		
F03	994		1959			1001									*accf*		
F04			2707			893									*cccf*		
F05	759		1358			680									*accf*		
F06	725		1361			731									*accf*		
F07	990		996			2164									*acff*		
F08			3468												*cccc*		
PLH08		523	866	730				672							*bcdh*		
G01	568		494			544		411							*acfh*	*af*	*ch*
G02	72			65		74		63							*adfh*	*af*	*dh*
G03	396	279	817												*abcc*	*ac*	*bc*
G04	811		881	739		770									*acdf*	*af*	*cd*
G05	667		1383	635											*accd*	*ac*	*cd*
G06	787	451	767					585							*abch*	*ac*	*bh*
G07			553	487		521		407							*cdfh*	*cf*	*dh*
G08	534	347	1010												*abcc*	*ac*	*bc*
PLF05	2336		1835												*aacc*		
H01	1191		1945			1060									*accf*		
H02	1105		3395												*accc*		
H03	1898		1667												*aacc*		
H04	1013		2933												*accc*		
H05	993		1856			995									*accf*		
H06	963		2898												*accc*		
H07	1991		706			905									*aacf*		
H08	1389		1413												*aacc*		
PHH02	652			498			222						554		*adgm*		
I01	3698												917		*aaam*	*aa*	*am*
I02	1007		979				391						850		*acgm*	*ac*	*gm*
I03	1696		573										712		*aacm*	*ac*	*am*
I04	2614		1115	1079											*aacd*	*ac*	*ad*
I05	1209		2122										941		*accm*	*cc*	*am*
I06			1704	833									773		*ccdm*	*cc*	*dm*
ZLH05		391			408						385			249	*bekn*		
K01	1272		1008		935						811				*acek*	*ac*	*ek*
K02	1040		875		811									653	*acen*	*ac*	*en*
K03		1005	1576								818				*bcck*	*cc*	*bk*
K04	788		738		718						690				*acek*	*ac*	*ek*
K05	980		902		775									627	*acen*	*ac*	*en*
K06	1005	980	807								730				*abck*	*ac*	*bk*

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	Allele peak height														Genotype		
Individual	094-a	096-b	098-c	100-d	102-e	104-f	105-g	106-h	108-i	110-j	112-k	114-l	116-m	118-n	129-o	Individual	Ovule	Pollen
PHH06			339	428	352								353			*cdem*		
A01			301	402	345								365			*cdem*		
A02			417	564	532								531			*cdem*		
A03			737	1989	826											*cdde*		
A04				2934	2138											*ddee*		
A05				2692	1907											*ddee*		
A06				1129	765								1898			*demm*		
A07			967		943								1818			*cemm*		
A08					4214								3445			*eemm*		
PHH01		1632			754		856									*bbeg*		
B01		844	629				628						542			*bcgm*	*cm*	*bg*
B02		1840	833										920			*bbcm*	*cm*	*bb*
B03		2989	2103		2044		2307									*bceg*	*ce*	*bg*
B04		3301	2911	4092	3086											*bcde*	*cd*	*be*
B05		1802	1674		1600		1799									*bceg*	*ce*	*bg*
B06		1919		2042	3264											*bdee*	*de*	*be*
PHF14	344		437	254	289											*acde*		
C01	66		223										143			*accm*	*ac*	*cm*
C02	302			609	367											*adde*	*ad*	*de*
C03	239		361	355									324			*acdm*	*a(c/d)*	*(c/d)m*
C04			602	259	294											*ccde*	*c(d/e)*	*c(d/e)*
C05				579	1059								583			*deem*	*de*	*em*
C06				937	1044											*ddee*	*de*	*de*
C07				1098	2106								1124			*deem*	*de*	*em*
C08			7635	8179												*ccdd*	*cd*	*cd*
PLH04	2803		(733)		1544			1792								*aaeh*		
D01	2630		(710)	3112	3087			2822								*adeh*	*d(a/e)*	*(a/e)h*
D02	650		796		778			638								*aceh*	*c(a/e)*	*(a/e)h*
D03	468		838		583			626								*aceh*	*c(a/e)*	*(a/e)h*
D04	1597		(414)		1833											*aaee*	*ae*	*ae*
D05	4173		2487													*aaac*	*ac*	*aa*
D06	2044		3356		2688			2489								*aceh*	*c(a/e)*	*(a/e)h*
D07			4359		8093			4017								*ceeh*	*ce*	*eh*
D08	6476		(1777)	3865	3584											*aade*	*de*	*aa*
ZHH18		1419		1478		1454										*bdf0*		
E01	3779	4419	4924	4128												*abcd*	*ac*	*bd*
E02	3439		4608	4241												*acd0*	*ac*	*d0*
E03	3180	3895	4615	3559												*abcd*	*ac*	*bd*
E04		2507	2521	4480												*bcdd*	*cd*	*bd*
E05	1477	2127	(243)	2247		2115										*abdf*	*ad*	*bf*
E06			3395	3578	3684											*cde0*	*ce*	*d0*
E07	2343		(238)	5842		2769										*addf*	*ad*	*df*
E08			3081	3150		3247										*cdf0*	*cd*	*f0*
PLH19	4035								6831	6840				6653		*aijn*		
F01									7667					7630		*iinn*		
F02	957								1563					2968		*ainn*		
F03	2331								4205	4316				4365		*aijn*		
F04									7065					6851		*iinn*		
F05										3424				3189		*jjnn*		
F06	2317								3860					7675		*ainn*		
F07									1867	900				821		*iijn*		
F08	486								1029					2132		*ainn*		
PLH08	1724	1750	(399)							1552	1672					*abjk*		
G01		1299							1244	1207				1210		*bijn*	*in*	*bj*
G02	77									77	65			57		*ajkn*	*(a/j)n*	*(a/j)k*
G03										1009	421			425		*jjkn*	*jn*	*jk*
G04	1213		(286)							2236				1120		*ajjn*	*jn*	*aj*
G05	1110		(246)						1114	2257						*aijj*	*ij*	*aj*
G06	1613		(389)							1693	1781			1602		*ajkn*	*(a/j)n*	*(a/j)k*
G07	1735		(287)							1104	1211					*aajk*	*aj*	*ak*
G08	1034									3336	1640					*ajjk*	*aj*	*jk*
PLF05	5826		(1433)							10480					5342	*ajjo*		
H01	3557		(858)							6945				3432		*ajjn*	*aj/jj*	*jn/an*
H02	1757		(296)							2725						*aajj*	*aj*	*aj*
H03	3350		(843)						3583	6607						*aijj*	*aj/jj*	*ij/ai*
H04									2605	5655					2743	*ijjo*	*jo*	*ij*
H05	4968		(1197)						5664					5625	4900	*aino*	*ao*	*in*
H06	4566		(1126)							9296				4973		*ajjn*	*aj/jj*	*jn/an*
H07	1817		(451)						2151	2083				2066		*aijn*	*aj*	*in*
H08	1143									2090				2225	2126	*ajno*	*ao/jo*	*jn/an*
PHH02	3061	4484	(795)			3380						3232				*abfl*		
I01	1952	1337	(443)							1004						*aabj*	*aj*	*ab*
I02	4488		(1090)									2014			1915	*aalo*	*ao*	*al*
I03	3635		(893)			3982				4033					3902	*afjo*	*jo*	*af*
I04		7902								6620		5277			5134	*bjlo*	*jo*	*bl*
I05	6943		(1640)			4021				4158						*aafj*	*aj*	*af*
I06		4246				3256				6736						*bfjj*	*jj*	*bf*
ZLH05		2754			2430	2902		2330								*befh*		
K01	4762	5470	(1296)		5169					4966						*abej*	*aj*	*be*
K02					6283	6992				6706					4941	*efjo*	*jo*	*ef*
K03		5945			5207					5518					4875	*bejo*	*jo*	*be*
K04		3518			3090					6605						*bejj*	*jj*	*be*
K05	4865		(1189)			6529		5398		5952						*afhj*	*aj*	*fh*
K06		6078				5797				5028					4394	*bfjo*	*jo*	*bf*

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	Allele peak height																					Genotype		
Individual	104-a	111-b	113-c	114-d	115-e	117-f	118-g	120-h	121-i	122-j	123-k	124-l	125-m	127-n	129-o	132-p	135-q	137-r	138-s	139-t	140-u	144-v	147-w	149-x	155-y	Individual	Ovule	Pollen
PHH06						1854						2040		1800		2054										*flnp*		
A01						1975						2063		1604		2130										*flnp*		
A02						1565						1460		1401		1536										*flnp*		
A03												3438		5466		3105										*lnnp*		
A04												6276		5984												*llnn*		
A05												3626		3311												*llnn*		
A06						4304						2383		1992												*ffln*		
A07						3993						1692				1701										*fflp*		
A08						4797						4800														*ffll*		
PHH01				1005							769					902					903					*dkpu*		
B01				604		667										1228										*dfpp*	*fp*	*dp*
B02				1330		1485						1015				1243										*dflp*	*fl*	*dp*
B03				2040								1966				3931										*dlpp*	*lp*	*dp*
B04												2136		2298		2202					2134					*lnpu*	*ln*	*pu*
B05											2052	1825				4311										*klpp*	*lp*	*kp*
B06				1984								1930		1717							1783					*dlnu*	*ln*	*du*
PHF14										1031					836						1019		908			*joux*		
C01						721				809						992							696			*fjpx*	*jx*	*fp*
C02										1305		1245		1339	1097											*jlno*	*jo*	*ln*
C03						1199				1078				1009									1067			*fjnx*	*jx*	*fn*
C04						868						850			778						887					*flou*	*ou*	*fl*
C05						1053						995			788						1028					*flou*	*ou*	*fl*
C06												1648		1652	1466						1806					*lnou*	*ou*	*ln*
C07						3477						3276			2281						3106					*flou*	*ou*	*fl*
C08														2327	2008	2379							2214			*nopx*	*ox*	*np*
PLH04				649				688												610					507	*dhty*		
D01								729							639					837	977					*hotu*	*ou*	*ht*
D02																				196	236		165	127		*tuxy*	*ux*	*ty*
D03																				136	130		121	90		*tuxy*	*ux*	*ty*
D04				391				363		392													363			*dhjx*	*jx*	*dh*
D05								472		474													434	323		*hjxy*	*jx*	*hy*
D06								842												799	1020		740			*htux*	*ux*	*ht*
D07				1774																1616	2219		1428			*dtux*	*ux*	*dt*
D08								2425							2052						2620			1593		*houy*	*ou*	*hy*
ZHH18			681		640								523									629				*cemv*		
E01			764		896					822													698			*cejx*	*jx*	*ce*
E02					747					860					640							732				*ejov*	*jo*	*ev*
E03			849		903					813													817			*cejx*	*jx*	*ce*
E04			1252		1268										1398								1356			*ceox*	*ox*	*ce*
E05			608							657			588		544											*cjmo*	*jo*	*cm*
E06													1210								1300	921	1010			*muvx*	*ux*	*mv*
E07					1480					1443			1275		1098											*ejmo*	*jo*	*em*
E08													1198		1148							1140	1115			*movx*	*ox*	*mv*

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	Allele peak height																						Genotype		
Individual	104-a	111-b	113-c	114-d	115-e	117-f	118-g	120-h	121-i	122-j	123-k	124-l	125-m	127-n	129-o	132-p	135-q	137-r	138-s	139-t	140-u	144-v	147-w	149-x	155-y	Individual	Ovule	Pollen
PLH19							2085						1907	1847												*gmn0*		
F01													6002													*mm00*		
F02													3659	1889												*mmn0*		
F03							1291						1344	1327												*gmn0*		
F04													2323													*mm00*		
F05							1890						1675													*ggmm/gm00*		
F06													692	344												*mmn0*		
F07							2966						2465													*ggmm/gm00*		
F08													3871	2165												*mmn0*		
PLH08									940									1059	1121				873			*irsw*		
G01													1855					1514					1312			*mrw0*	*m0*	*rw*
G02							149		123				124						108							*gims*	*gm*	*is*
G03							835						758					658	670							*gmrs*	*gm*	*rs*
G04							1715		1328				1446						1376							*gims*	*gm*	*is*
G05							1275											1009					918			*grw0*	*g0*	*rw*
G06							1817		1434				1540						1414							*gims*	*gm*	*is*
G07							1322		1025					1285					1098							*gins*	*gn*	*is*
G08							1575							1436				1402	1432							*gnrs*	*gn*	*rs*
PLF05						3256						2932				2999			2674							*flps*		
H01							2390						2032			2289			1973							*gmps*	*ps*	*gm*
H02							2315							2161		2179			1932							*gnps*	*ps*	*gn*
H03							1706									1407			1409							*gps0*	*ps*	*g0*
H04						881	908					791														*fgl0*	*fl*	*g0*
H05						2054							1743						1936							*fms0*	*fs*	*m0*
H06							1494					1406	1387						1407							*glms*	*ls*	*gm*
H07												827	830						856							*lms0*	*ls*	*m0*
H08						342						391	270	265												*flmn*	*fl*	*mn*
PHH02		2601											2393		2210				2385							*bmos*		
I01		827										792			648				699							*blos*	*ls*	*bo*
I02		2113				2278													3865							*bfss*	*fs*	*bs*
I03		1116				1137							964			922										*bfmp*	*fp*	*bm*
I04															2078	2116			4209							*opss*	*ps*	*os*
I05		1783														1506			2904							*bpss*	*ps*	*bs*
I06												893	991		828	850										*lmop*	*lp*	*mo*
ZLH05	1278					940				1049							1013									*afkq*		
K01	2552											2275					1842		2403							*alqs*	*ls*	*aq*
K02						2788				2459						2828	2518									*fkpq*	*fp*	*kq*
K03	1571					1784										1668	1489									*afpq*	*fp*	*kq*
K04	1070											1013				1179	997									*alpq*	*lp*	*aq*
K05						1694				2016						1579			1287							*fkps*	*ps*	*fk*
K06	1320					1424				1341						1231										*afkp*	*fp*	*ak*

Locus E070

	Allele peak height											Genotype		
Individual	128-a	132-b	134-c	136-d	138-e	140-f	142-g	144-h	145-i	146-j	148-k	152-l	Individual	Ovule	Pollen
PHH06	464	908	374										*abbc*		
A01	844	848											*aabb*		
A02	593	1153	455										*abbc*		
A03	1121	2149	827										*abbc*		
A04	1352	2588	975										*abbc*		
A05	2681	1575	1048										*aabc*		
A06	2058	2068											*aabb*		
A07	2300	2236											*aabb*		
A08	1685	1894	2475										*abcc*		
PHH01				668	945				437				*deei*		
B01	378		282		340				260				*acei*	*ac*	*ei*
B02		1687		805	767								*bbde*	*bb*	*de*
B03		3356		1496					1278				*bbdi*	*bb*	*di*
B04	1590	1410		1342	1263								*abde*	*ab*	*de*
B05	1112	1137		941	873								*abde*	*ab*	*de*
B06		1095	714		824				694				*bcei*	*bc*	*ei*
PHF14				384	459	383			293				*defi*		
C01	131	138		125					95				*abdi*	*di*	*ab*
C02		454	345	399	420								*bcde*	*de*	*bc*
C03		349	288	293					256				*bcdi*	*di*	*bc*
C04		397	321			357			262				*bcfi*	*fi*	*bc*
C05	660	701		599		594							*abdf*	*df*	*ab*
C06	649	593		603	566								*abde*	*de*	*ab*
C07	1082		843		1071	856							*acef*	*ef*	*ac*
C08	1140	1081		901					732				*abdi*	*di*	*ab*
PLH04		575			643	583		386					*befh*		
D01				417	456	385			295				*defi*	*di*	*ef*
D02		182		187	213	177							*bdef*	*d(e/f)*	*b(e/f)*
D03		155		177				105	100				*bdhi*	*di*	*bh*
D04		520		473		508		323					*bdfh*	*df*	*bh*
D05				538	597	898							*deff*	*df*	*ef*
D06		635			707	596			491				*befi*	*(e/f)i*	*b(e/f)*
D07				633	636	564			454				*defi*	*di*	*ef*
D08					1003	482			395				*eefi*	*ei*	*ef*
ZHH18					658					312	252		*eejk*		
E01				375	323				323		292		*deik*	*di*	*ek*
E02				345	365				277		250		*deik*	*di*	*ek*
E03					935				390		395		*eeik*	*ei*	*ek*
E04				548	517	501				426			*defi*	*df*	*ej*
E05				654	582				479		419		*deik*	*di*	*ek*
E06					979				468	431			*eeij*	*ei*	*ej*
E07					1123	537				448			*eefi*	*ef*	*ej*
E08				508	834						302		*deek*	*de*	*ek*
PLH19		1204		1171	2033								*bdee*		
F01		1026			2548								*beee*		
F02		1148		2035	979								*bdde*		
F03		1079			2778								*beee*		
F04				1006	2355								*deee*		
F05				1505	1406								*ddee*		
F06				1476	1408								*ddee*		
F07		1197		2017	972								*bdde*		
F08		917		734	1379								*bdee*		
PLH08				1375	2113			1139					*deeh*		
G01		951			1534			667					*beeh*	*be*	*eh*
G02		58			84			42					*beeh*	*be*	*eh*
G03		538		458	408			382					*bdeh*	*b(d/e)*	*(d/e)h*
G04				1238	1989			863					*deeh*	*ee/de*	*dh/eh*
G05				886	1577			670					*deeh*	*ee/de*	*dh/eh*
G06				1239	2231			938					*deeh*	*ee/de*	*dh/eh*
G07		880			1354			619					*beeh*	*be*	*eh*
G08		1094		963	1667								*bdee*	*bd/be*	*ee/de*
PLF05		2195		1660									*bbdd*		
H01		1150		1945	965								*bdde*	*d(b/d)*	*(b/d)e*
H02		962		1171	2035								*bdee*	*bd*	*ee*
H03		2426		1834									*bbdd*	*bd*	*bd*
H04		1864		864	864								*bbde*	*b(b/d)*	*(b/d)e*
H05		3039			974								*bbbe*	*bb*	*be*
H06		1768		962	957								*bbde*	*b(b/d)*	*(b/d)e*
H07		978		1145	2035								*bdee*	*bd*	*ee*
H08		2591			777								*bbbe*	*bb*	*be*
PHH02				327		612	272						*dffg*		
I01		2032		923		973							*bbdf*	*bb*	*df*
I02		936		799		935	652						*bdfg*	*bd*	*fg*
I03		666		705		840	614						*bdfg*	*bd*	*fg*
I04				3434			1055						*dddg*	*dd*	*dg*
I05		1152		954		1042	800						*bdfg*	*bd*	*fg*
I06		839		1567		836							*bddf*	*bd*	*df*
ZLH05					382			164				157	*eehl*		
K01		743		647	613							413	*bdel*	*bd*	*el*
K02				1582	728			587					*ddeh*	*dd*	*eh*
K03		733		823	802							558	*bdel*	*bd*	*el*
K04				1353	687							542	*ddel*	*dd*	*el*
K05		1676			855			659					*bbeh*	*bb*	*eh*
K06		1022		815	809							560	*bdel*	*bd*	*el*

Locus E089

	Allele peak height				Genotype		
Individual	124-a	127-b	130-c	133-d	Individual	Ovule	Pollen
PHH06			2492		*cccc*		
A01			3840		*cccc*		
A02			5167		*cccc*		
A03			7697		*cccc*		
A04			6040		*cccc*		
A05			7726		*cccc*		
A06			6875		*cccc*		
A07			7403		*cccc*		
A08			6388		*cccc*		
PHH01	617		1828		*accc*		
B01			1015		*cccc*	*cc*	*cc*
B02	681		2072		*accc*	*cc*	*ac*
B03	1568		4692		*accc*	*cc*	*ac*
B04	1937		5795		*accc*	*cc*	*ac*
B05			4532		*cccc*	*cc*	*cc*
B06			5053		*cccc*	*cc*	*cc*
PHF14	763		2285		*accc*	*ac*	*cc*
C01	529		1471		*accc*	*ac*	*cc*
C02	616		1975		*accc*	*ac*	*cc*
C03			3381		*cccc*	*cc*	*cc*
C04			1890		*cccc*	*cc*	*cc*
C05	1260		3778		*accc*	*ac*	*cc*
C06	1300		3879		*accc*	*ac*	*cc*
C07	1631		5480		*accc*	*ac*	*cc*
C08	1479		4895		*accc*	*ac*	*cc*
PLH04			3114		*cccc*		
D01			3578		*cccc*	*cc*	*cc*
D02			773		*cccc*	*cc*	*cc*
D03	281		664		*accc*	*ac*	*cc*
D04			2349		*cccc*	*cc*	*cc*
D05	607		1764		*accc*	*ac*	*cc*
D06			4733		*cccc*	*cc*	*cc*
D07	1168		3943		*accc*	*ac*	*cc*
D08			5145		*cccc*	*cc*	*cc*
ZHH18		379	1371	533	*bccd*		
E01			1526	486	*cccd*	*cc*	*cd*
E02	693	456	1745		*abcc*	*ac*	*bc*
E03	1095	681	2658		*abcc*	*ac*	*bc*
E04			2993		*cccc*	*cc*	*cc*
E05			3249	976	*cccd*	*cc*	*cd*
E06	1334	820	3381		*abcc*	*ac*	*bc*
E07			4426	1378	*cccd*	*cc*	*cd*
E08	1226		4096		*accc*	*ac*	*cc*
PLH19	1517		5315		*accc*		
F01	1558		5910		*accc*		
F02			6578		*cccc*		
F03			5814		*cccc*		
F04	2064		2340		*aacc*		
F05	876		3152		*accc*		
F06	903		3222		*accc*		
F07	1416		5101		*accc*		
F08	2004		5997		*accc*		
PLH08			4107	1378	*cccd*		
G01			4242		*cccc*	*cc*	*cc*
G02			337	104	*cccd*	*cc*	*cd*
G03	749		1428	685	*accd*	*ac*	*cd*
G04	1628		4571		*accc*	*ac*	*cc*
G05	1185		2033	937	*accd*	*ac*	*cd*
G06	1172		2032	1008	*accd*	*ac*	*cd*
G07	1101		2079	965	*accd*	*ac*	*cd*
G08			4330		*cccc*	*cc*	*cc*
PLF05	2975		3519		*aacc*		
H01	937		3246		*accc*		
H02	3397		3732		*aacc*		
H03	4498		1754		*aaac*	*(aa)*	*(ac)*
H04	1344		4650		*accc*		
H05	1176		4032		*accc*		
H06	4027		1503		*aaac*	*(aa)*	*(ac)*
H07	1205		4152		*accc*		
H08	1915		2242		*aacc*		
PHH02	950		3997		*accc*		
I01	3180		3762		*aacc*		
I02	2266		2543		*aacc*		
I03	904		2990		*accc*		
I04	1642		6118		*accc*		
I05	1314		4911		*accc*		
I06	1042		3814		*accc*		
ZLH05	769		2305		*accc*		
K01	3688		4146		*aacc*		
K02	2968		3431		*aacc*		
K03	1073		3682		*accc*		
K04	805		2749		*accc*		
K05	3291		1307		*aaac*	*(aa)*	*(ac)*
K06	1194		4028		*accc*		

Chapter 3

Does the outcrossing advantage of females increase with increasing altitude in tetraploid gynodioecious *Thymus praecox* agg.?

Urs Landergott

Abstract

The avoidance of inbreeding is often considered a driving force in the evolution and maintenance of sexual dimorphism in flowering plants. In gynodioecious (coexistence of females and hermaphrodites) *Thymus praecox* from the Swiss Alps, the frequency of females increases with increasing elevation, which parallels expectations of increased rates and/or costs of self-fertilisation (i.e. the two major determinants of an outcrossing advantage) under harsh alpine conditions. Here I use highly variable co-dominant microsatellite markers to explore the major determinants of the geitonogamous selfing rate of hermaphrodites, as inferred from genetic analysis of their open-pollinated offspring, and the costs of selfing, as inferred from a comparison of heterozygosity of selfed offspring and of adult populations of tetraploid *T. praecox* agg. from four subalpine and four alpine sites. Selfing rates of individual hermaphrodites (mean = 0.39) were significantly affected by local neighbourhood hermaphrodite density, irrespective of altitude. In contrast, no significant effect of floral display size of focal hermaphrodites on their geitonogamous selfing rate was detected, except for a few largest individuals from low altitudes. There was no evidence of a shift in pollinator-mediated selfing across altitudes, and mean selfing rates per population did also not generally differ among altitudes. The average heterozygosity of selfed offspring was significantly lower than the heterozygosity of adult populations, and the results suggested that selfed offspring largely fail to reach reproductive maturity in natural populations of *T. praecox*, indicating a substantial outcrossing advantage independent of altitude. These findings rejected the hypothesis that alpine conditions altered the relative seed fitness of the two sexual phenotypes due to selfing in hermaphrodites, as compared with subalpine sites. The significant outcrossing advantage of females did thus not explain the observed sex ratio variation along the elevation gradient in *T. praecox*.

Key words: altitudinal gradient, floral display size, geitonogamy, heterozygosity, inbreeding avoidance, local neighbourhood structure, nuclear-cytoplasmic gynodioecy, sex ratio

Introduction

Ecological correlates point to a significant role of habitat shifts for the evolution of gender dimorphism (separate sexes) in flowering plants, for instance through changes in pollination biology and realised mating system (Sakai & Weller, 1999; Barrett, 2003). Shifts in pollinator assemblage, abundance or behaviour may alter outcrossing rates and, depending on the cost of self-fertilisation, generate selection for avoidance of inbreeding and thereby favour unisexual individuals (Charlesworth, 1999). Species that exhibit intraspecific sexual system variation along ecological gradients provide attractive model systems to study potential triggers for the evolution and maintenance of gender dimorphism (Webb, 1999).

In *Sagittaria latifolia*, the finding of significantly higher rates of self-fertilisation in monoecious than in dioecious populations has been interpreted as evidence that inbreeding avoidance was involved in the transition from monoecy to dioecy (Dorken *et al.*, 2002), which may have been stimulated by a shift from frequently disturbed to more stable habitats, which affected clone size and the rate of geitonogamous selfing (Barrett, 2003). In *Limnanthes douglasii* (Kesseli & Jain, 1984) and *Wurmbea biglandulosa* (Ramsey *et al.*, 2006b) hermaphrodite individuals have been shown to experience substantial selfing in gynodioecious populations (coexistence of females and hermaphrodites), but not in monomorphic populations that contain hermaphrodites only. Ramsey *et al.* (2006b) proposed that gynodioecy in *W. biglandulosa* was favoured to avoid inbreeding under conditions that promote pollinator-mediated selfing. In *Hebe*, Delph (1990) found that the degree of gender dimorphism was positively correlated with altitude and hypothesized that changes in pollinator assemblage and reduced pollination efficiency increased selfing at higher altitudes, which promoted the evolution of gynodioecy (but see Maurice & Fleming, 1995). Increased frequencies of females at higher altitudinal alpine sites have also been observed within several gynodioecious species (Schrader, 1986; Alatalo & Molau, 1995; Puterbaugh *et al.*, 1997; Landergott *et al.*, submitted), which raises the question whether inbreeding avoidance may cause sex ratio variation along such elevation gradients in gynodioecious species.

Evidence of an outcrossing advantage of females as compared with hermaphrodites has been detected in many self-compatible gynodioecious species (Webb, 1999). Costs of self-fertilisation may vary among populations and among environments (Perrot *et al.*, 1982; Koelewijn, 1998; Thompson & Tarayre, 2000), and their magnitude under natural conditions may be substantial as inferred from estimates based on genetic markers (Kohn & Biardi, 1995; Sakai *et al.*, 1997; Medrano *et al.*, 2005; Ramsey *et al.*, 2006a, b). If selfed offspring largely fail to reach reproductive maturity, variation in the rate of self-fertilisation of hermaphrodites will primarily determine the outcrossing advantage of females. Selfing rates of hermaphrodites in insect pollinated gynodioecious species are often significant (reviewed in Collin & Shykoff, 2003), vary among individuals within populations (Valdeyron *et al.*, 1977; Wolff *et al.*, 1988) and may be determined by the density of outcross pollen donors at small spatial scales (Brabant *et al.*, 1980; van Treuren *et al.*, 1993; Garcia *et al.*, 2005). As a consequence, population density and female frequency will also affect the average selfing rate of hermaphrodites in a

population (Sun & Ganders, 1988; Wolff *et al.*, 1988; van Treuren *et al.*, 1993). Ecological factors that alter either component (i.e. the cost or the rate of self-fertilisation) of the outcrossing advantage of females may alter the relative seed fitness of the two sex types and consequently the equilibrium sex ratio within populations (Charlesworth, 1999).

Along subalpine to alpine altitudinal gradients, decreasing temperature and growing season length (Körner, 1999) could affect both the cost and rate of self-fertilisation. Putatively, increasing habitat harshness and extended time to reproductive maturity could restrict the chance of inbred progeny to reproduce at higher altitudes (von Arx *et al.*, 2006). Furthermore, hostile abiotic conditions, shifts in pollinator fauna and reduced flower visitation rates at higher altitudes (Müller, 1880; Arroyo *et al.*, 1985; Bingham & Orthner, 1998) could alter pollinator behaviour and the extent of geitonogamous selfing (Goulson, 1999; Herrera, 2000; Utelli & Roy, 2000; Brunet & Sweet, 2006). However, while classical views on pollen limitation in arctic-alpine environments have been challenged by recent empirical work (Kearns & Inouye, 1994; Bingham & Orthner, 1998; Gugerli, 1998; Totland & Schulte-Herbruggen, 2003), potential effects of alpine conditions on the effectively realised mating system still remain largely unexplored within species (Arroyo *et al.*, 2006).

The gynodioecious *Thymus praecox* agg. from the Swiss Alps provides an opportunity to examine the effects of ecological conditions on outcrossing advantage and equilibrium sex ratio in this species. The proportion of hermaphrodites decreases with increasing altitude in natural populations of *T. praecox* agg. from the Swiss Alps (Landergott *et al.*, submitted). Offspring sex ratios suggest that hermaphrodites contribute less to the next generation via their female reproductive function at higher than at lower altitudes (Landergott *et al.*, submitted). The species is insect-pollinated and shows large floral displays; geitonogamous selfing in hermaphrodites may thus be expected (de Jong *et al.*, 1993; Karron *et al.*, 2004).

Here I test the hypothesis that differences in the outcrossing advantage of females among populations influence sex ratio variation across subalpine to alpine elevation gradients in the gynodioecious *T. praecox* agg. I compare low and high altitudinal natural populations of *T. praecox* agg. replicated over four different valleys of the Swiss Alps. I use highly variable microsatellite markers to estimate rates of geitonogamous selfing, and I relate selfing rates of individual hermaphrodites to floral display size and small-scale availability of potential outcross-pollen donors in order to explore mating patterns at contrasting altitudes. Furthermore, I estimate the relative magnitude of costs of self-fertilisation at contrasting altitudes from levels of heterozygosity among adult populations and among open-pollinated offspring. An increase in the rate and/or the cost of selfing with increasing altitude will indicate a role of inbreeding avoidance for the maintenance of sex ratio variation among the gynodioecious study populations.

Materials and methods

Study species and populations

Thymus praecox agg. Opiz ampl. Jalas (Lamiaceae) is a tetraploid with 2*n* = (50-) 56 (Jalas, 1970; Landergott *et al.*, 2006). The species is widespread in the European Alps from subalpine to alpine altitudes and is found on rocky surfaces, in pastures and alpine grassland. It is a long-lived perennial forming carpets with densely arranged inflorescences harbouring numerous, small purple flowers (Tutin *et al.*, 1972). Hermaphrodites are self-compatible, but their flowers are highly protandrous and rely on insect visitors for pollen transfer (Landergott *et al.*, submitted). The unspecialised insect visitors readily crawl from flower to flower and from inflorescence to inflorescence (U. Landergott, pers. observ.). Thyme produces four ovules per flower.

In this study, I compared four subalpine and four alpine populations of *T. praecox* agg. from four different valleys in the Swiss Alps (Table 1). Population abbreviations indicate the study region (L = Langwies, P = Piora, S = Säntis, Z = Zwinglipass) and the altitude (L = low, H = high); abbreviation LH thus refers to the high altitude study population form the region Langwies (see Landergott *et al.* (submitted) for precise locations). *Thymus praecox* agg. was abundant and widely distributed within the study regions, and the study sites (300 to 400 m^2 each) had similar slopes and southern exposition. Alpine sites are characterised by lower average temperature during the vegetation period and higher humidity as compared to subalpine sites (Landergott *et al.*, submitted). *Thymus praecox* agg. flowered from June to July at low altitude and from July to August at high altitude sites, and the two sexual phenotypes were patchily distributed within populations (Landergott *et al.*, submitted). I did not conduct systematic pollinator observations in the present study. However, honeybees appeared to be the most common flower visitor of thyme at subalpine sites, and solitary bees, bee-flies, syrphid flies, muscoid flies and butterflies regularly foraged on thyme at low altitudes. Bumblebees preferred visiting other plant species at low altitudes. In contrast, bumblebees frequently foraged on thyme at high altitudes, and syrphid flies and muscoid flies were also common visitors at alpine sites. Butterflies were regularly visiting thyme at alpine sites as well, but bee species were seen at alpine sites only during prolonged periods of good and warm weather conditions, but still in low abundance. However, there seemed to be substantial variation in the relative abundance of pollinator types among populations within altitudes. Finally, flower visitations appeared to be concentrated in the first sunny hours of a day at low altitudes, whereas visitation activity at high altitudes was more evenly distributed over the day (U. Landergott, pers. observ.)

Within each of the eight study populations, 15-20 individuals of *T. praecox* agg. displaying a minimum of five inflorescences were randomly chosen as focal plants and permanently marked, as well as the nearest neighbour of the complementary sexual phenotype (Landergott *et al.*, submitted). In the years 2001 (populations PH and PL), 2002 (LH, SL and ZL) and 2003 (LL, SH and ZH), the number of inflorescences was recorded per focal plant

(adults) and leaf material was collected and dried on silica gel. In addition, I determined the neighbourhood of each focal plant by recording the distance to the closest six individuals of *T. praecox* agg., their sexual phenotype and their number of inflorescences. Later on, ripe fruits were collected from the focal plants and seeds were sown in a greenhouse at the Botanical Garden of Zurich (Landergott *et al.*, submitted). Per hermaphrodite mother plant, up to ten seedlings (offspring) were randomly collected and silica dried.

Controlled crosses on hermaphrodites (treatments HS for self-pollination and HO for within population outcrosses with one hermaphrodite pollen donor per cross; Landergott *et al.*, submitted) were used to examine the effects of selfing on seed production and germination. The sample set existed of 54 crosses: 27 maternal half-sib families that comprised both cross treatments from populations PH (n = 6), PL (n = 8), ZH (n = 6) and ZL (n = 7) (two half-sib families were excluded due to contaminated offspring; Landergott *et al.*, submitted) and another two half-sib families where a hexaploid individual (see below) was involved were also omitted). I recorded the seed set of 15 randomly sampled fruits per cross. Seeds were sown in the greenhouse at the Botanical Garden of Zurich under the same conditions as the above-mentioned open-pollinated offspring, and the germination ratio (proportion germinated seeds) was determined per cross family (Landergott *et al.*, submitted).

Microsatellite markers

For molecular genetic analyses, I used a set of six nuclear, highly variable and co-dominant microsatellite markers that have been characterised in Landergott *et al.* (2006). Briefly, high levels of allelic diversity and heterozygosity at the six microsatellite loci permit to directly discriminate between outcrossed and selfed offspring in natural populations of *T. praecox* agg. Allele copy numbers per individual and locus are resolved by PCR product intensity; the different heterozygosity states that occur under tetrasomic inheritance can thus be appropriately scored. Controlled crosses have demonstrated Mendelian tetrasomic inheritance with nearly random chromosome segregation (i.e. no evidence of frequent double reduction has been detected).

I genotyped 15-20 adult individuals of *T. praecox* agg. per sex type and population following the procedures described in Landergott *et al.* (2006). Due to the dense and admixed growth of adult plants, I expected to find mis-assigned offspring in some of the sampled families of open-pollinated hermaphrodites; five families had to be omitted for this reason. For 81 remaining hermaphrodite seed-parents, I genotyped up to seven seedlings (mean = 6.7) per family. Another 6% of these seedlings turned out to be contaminants that were omitted from the data set and replaced by additional true half-sibs. Copy numbers of alleles at a given microsatellite locus were determined from electropherogram peak heights of fragment analyses using GENESCAN ver. 3.7 (Applied Biosystems; Landergott *et al.*, 2006), and allelic configurations were recorded manually. Two adult individuals from populations SL and ZH displayed a hexaploid allelic configuration at all six loci and were omitted from the data set (the hexaploid pattern was reproducible across independently extracted templates). Triploid

allelic configurations were found in 2.4% of the adult individuals at locus C405, 3.1% at D257, 7.7% at D346, 4.2% at D347, 2.4% at E070 and 2.1% at E089. I interpreted these triploid patterns as incomplete allelic configurations caused by putative null alleles, since their frequencies corresponded to the frequencies of mutations detected in the flanking-sequences of the six microsatellite loci (Landergott *et al.*, 2006).

Statistical analysis

I scored the observed heterozygosity (H_O) at individual loci by weighting the five possible classes of genotypes by 1 minus the probability of any two alleles taken at random from an individual being identical by descent: *AAAA* = 0, *AAAB* = 1/2, *AABB* = 2/3, *AABC* = 5/6 and *ABCD* = 1 (H_O = 1 – the individual inbreeding coefficient; Bever & Felber, 1992).

A first set of analyses aimed at further exploring characteristics of the six microsatellite loci as relevant to marker interpretation. No common method was available to test for genetic linkage disequilibrium between the loci. I thus calculated pairwise Pearson correlations between single locus-heterozygosities (H_O) among the adult individuals of *T. praecox* agg. separately for each population, and determined the average correlation coefficients of each pair of loci among populations. In addition, within each of the three populations LH, SL and ZH, for each pair of loci, I tested for non-random associations between the most frequent alleles by constructing 2 × 2 contingency tables with the number of individuals in each combination of genotypes (presence or absence of the most frequent allele; Fisher's Exact test; Flajoulot *et al.*, 2005). I also plotted the allele frequencies per locus and population in both sexual phenotypes in order to visualize potential sex linkage. Furthermore, under nuclear-cytoplasmic sex determination, proximity of a marker locus to a locus involved in the restoration of the male function could lead to differing heterozygosities between the two sex types (Charlesworth & Laporte, 1998; Hansson & Westerberg, 2002). I used generalised linear models (GLMs; with binomial error distribution, logit link function and adjustment for overdispersion; SAS Institute, 2005) to check for effects of sex, population and their interaction on locus-specific H_O among adults. Finally, I compared the six loci with respect to their conformity to Hardy-Weinberg equilibrium. I used the software AUTOTET (Thrall & Young, 2000) to calculate expected heterozygosities (H_E) and population inbreeding coefficients ($F_{IS} = 1 - H_O/H_E$) under random chromosome segregation, for adult hermaphrodites [h] and females [f] separately. Values of F_{IS} were normally distributed with only four outliers from locus E089. Because there was no obvious relationship with sex type or altitude, I interpreted these outliers as being attributable to the relatively low allelic diversity at this locus (Thrall & Young, 2000). After having confirmed that values of F_{IS} did not differ between the two sex types (paired *t*-tests, $P > 0.05$ for all loci), I calculated the average H_E and F_{IS} of the two sex types per locus and population for further analyses (two estimates of F_{IS} at locus E089 remained outliers [LH: F_{IS} = 0.102; SL: F_{IS} = -0.087] and were excluded from the following ANOVA as well as from the calculation of mean F_{IS} per population; Table 1).

To test for differences in adult population inbreeding coefficients among altitudes and, simultaneously, between loci, I performed an ANOVA on locus and population specific F_{IS} with the three factors altitude, locus and region (all two-way interactions were not significant with $P \geq 0.25$ and omitted from the final analysis). A similar ANOVA model was used to test for an effect of altitude on the allelic richness of adult populations, with locus, region and all two-way interactions as random effects. For this purpose, estimates of the number of alleles per locus were standardized for a fixed sample size of 26 individuals per population by means of rarefaction using ANALYTIC RAREFACTION ver. 1.3 (http://www.uga.edu/strata/software; El Mousadik & Petit, 1996). To examine whether the degree of population structure did vary with altitude, I randomly selected either the female or the hermaphrodite of each pair of neighbours and determined its genetic similarity with two individuals from two different distance classes, with its nearest neighbour of the complementary sex and with an individual taken at random from the population. I determined the similarity per locus as the number of shared alleles (Kosman & Leonard, 2005), weighted these values by population and locus specific H_E (the relative information content of the loci) and summed them to obtain a multilocus similarity estimate. A partly nested ANOVA (Quinn & Keough, 2002) was performed on square root-transformed genetic similarities to test for an effect of altitude (fixed), region (random) and their interaction (between subjects factors), with individual comparisons nested within altitude and region as the denominator for the test of altitude, and to test for an effect of distance class (fixed) and its interactions with altitude and region (within subjects factors; $n = 134$ individual comparisons). Finally, two ANOVAs on the number of inflorescences in hermaphrodites (floral display size; $n = 342$; ln-transformed) and on the average spatial distance between each focal hermaphrodite and the hermaphrodites among its six nearest neighbours (average spatial distance; $n = 80$, one spatially isolated individual from population SH causing an extreme outlier residual was omitted) were applied to test for differences among altitudes (fixed), with region and altitude × region as random effects.

I applied two partly nested ANOVAs to test for effects of self-fertilisation on mean seed set per fruit and on germination ratio (arcsine-transformed). These ANOVAs comprised altitude as a fixed factor and region, the interaction between altitude and region and mother nested within altitude × region as random effects, the cross treatment as the fixed effect of primary interest and, for seed set, its interaction with the random factor region (nonsignificant two and three way interactions between cross treatment, altitude and region with $P \geq 0.35$ were omitted from the final analyses).

To determine whether seedlings of open-pollinated hermaphrodite mothers originated from self-pollination or from outcrossing, I compared their genotype with their maternal genotype for each locus separately, recorded outcross- vs. potential self-fertilisation events per locus and concatenated the records across loci. Offspring lacking any indication of an outcrossing event (97% of the outcrosses were denoted by at least two different loci) were designated selfs, and selfing rates were calculated per open-pollinated maternal half-sib family. Based on the results from the controlled crosses (see below), I used the unadjusted selfing rates

in seedlings (Maki, 1993) for further analyses. The effect of altitude on the rate of self-fertilisation was tested in an ANOVA over the random factor population nested within altitude. In the case of pollinator-mediated geitonogamous self-fertilisation, individual selfing rates are expected to covary with floral display size (de Jong *et al.*, 1993) and with the spatial proximity and the size of potential outcross pollen donors (I used a compound predictor: neighbourhood pollen availability = $\sum$ spatial distance$^{-0.75}$ × floral display size of hermaphrodite neighbour; Brabant *et al.*, 1980; van Treuren *et al.*, 1993; Goulson, 1999). I was primarily interested in potential differences in the relationship between the two predictor variables and selfing rates across altitudes, a question calling for ANCOVA. However, because the value ranges of the two predictor variables were not congruent among altitudes, I decided to first explore the effects of the two variables on selfing rates within altitudes, and to subsequently reduce the data set according to the predictor that would be of most interest in an among altitude ANCOVA. Floral display size and neighbourhood pollen availability were positively correlated within altitudes (low altitude: $n = 38$; $r = 0.30$, $P = 0.074$; high altitude: $n = 43$; $r = 0.49$, $P = 0.001$), variance inflation factors (< 3.5 in all cases) indicated though no collinearity problems in the following analyses. Furthermore, scatterplots between the two predictor variables and selfing rates revealed no evidence of nonlinear relationships. Within the two altitude levels, I fitted a separate model each with the two continuous predictors floral display size and neighbourhood pollen availability, with population as a blocking factor and the interactions between the two continuous predictors and population. Nonsignificant ($P > 0.20$) effects of predictor × population interactions and of population were backward excluded. In the high altitude model, however, exclusion of the neighbourhood pollen availability × population interaction would have caused a drop in adjusted R-squared by one half and led to a skewed pattern in the residual plot (one individual was omitted from the final analysis as it consistently displayed outlier residuals and unusual effect leverages). Based on the results of within altitude analyses, I restricted the data set to focal hermaphrodites showing five to 100 inflorescences (i.e. the main range of floral display size at high altitude; 16 larger individuals were excluded) and tested for an altitude × floral display size interaction using ANCOVA (also accounting for the effects of altitude, floral display size, neighbourhood pollen availability and its interaction with altitude). Finally, I further reduced the data set to ensure overlapping ranges of neighbourhood pollen availability across altitudes as well, as this reduced data set appeared most reliable to test for differences in selfing rates caused by differences in pollinator behaviour per se. I performed a *t*-test assuming unequal variances for an effect of altitude on selfing rates (data pooled across regions; low altitude: $n = 16$; high altitude: $n = 36$).

Inbreeding increases homozygosity, with an expected loss of heterozygosity of 0.17 per generation of selfing under tetrasomic inheritance and random chromosome segregation (Bever & Felber, 1992). In partially selfing species, the chance of inbred offspring to reproduce under natural conditions can thus be estimated from comparisons of heterozygosity between adults and their offspring (Ritland, 1990). To compare levels of observed heterozygosity among individuals, I calculated a weighted average observed multilocus heterozygosity ($wMLH_O$) per

individual, with the average of H_O weighted by population and locus specific H_E (the relative information content of the loci). Arcsine-transformed $wMLH_O$ of adult females and hermaphrodites was analysed in a partly nested ANOVA to test for an effect of altitude (fixed), region (random) and their interaction, using pairs of nearest neighbours nested within altitude and region as denominator for altitude, and to test for a difference between sex types (fixed) and an interaction with altitude (sex type × region and altitude × region were not significant with $P > 0.50$ and omitted from the final model). For the comparison of adult and offspring heterozygosities, I was primarily interested in variation in the magnitude of a potential difference between generations across altitudes since such an interaction effect would point to differing costs of inbreeding at contrasting altitudes. I performed two separate analyses to compare heterozygosity of adults and of selfed seedlings (indicative of costs of self-fertilisation) and heterozygosity of adults and of outcrossed seedlings (potentially indicative of biparental inbreeding). ANOVAs on arcsine-transformed $wMLH_O$ were used to test for interactions between altitude and generation, in models comprising the fixed factors altitude and generation, the random effect of region and all two-way interactions. However, solely analysing means may be misleading, because high levels of heterozygosity will be maintained in a part of the selfed offspring under tetrasomic inheritance (Bever & Felber, 1992). Therefore, I also determined the ranges of heterozygosity values lying within the 5-95% quantiles for adults, selfed and outcrossed offspring per population. I recorded the proportion of selfed and outcrossed offspring showing heterozygosity estimates that were lower than the range of adult heterozygosities per population. Separately for selfed and outcrossed offspring, I applied logistic regression analyses to test for effects of altitude and region on the proportion of individual offspring that were less heterozygote than the least heterozygote adults per population. All statistical analyses were performed in JMP ver. 6.0 (SAS Institute Inc, Cary, NC, USA; Quinn & Keough, 2002; SAS Institute, 2005).

Results

Microsatellite markers

Among a total of 286 genotyped adult individuals from eight populations of *T. praecox* agg., a total of 62 different alleles (A_T) were detected at the most variable (compound) microsatellite locus D347, on average 34.8 per population (standardised for 26 individuals; A_{26}), with an average of 3.7 different alleles per individual (A_I). The corresponding estimates of allelic diversity were $A_T = 36$, $A_{26} = 22.5$ and $A_I = 3.6$ for locus D257, $A_T = 36$, $A_{26} = 17.8$ and $A_I = 3.5$ for locus D346, $A_T = 26$, $A_{26} = 15.7$ and $A_I = 3.4$ for locus C405, $A_T = 29$, $A_{26} = 13.8$ and $A_I = 3.1$ for locus E070 and $A_T = 10$, $A_{26} = 6.5$ and $A_I = 2.3$ for locus E089, respectively. Correlations between observed heterozygosities at different loci were weak, with the correlation coefficients averaged across populations ranging from $r = -0.1$ between loci D346 and D347 to $r = 0.1$ between C405 and D346, and with maximal within population pairwise correlations of $r = 0.4$ (only statistically significant if not adjusted for multiple comparisons).

Within populations LH, SL and ZH, presence and absence of the most frequent alleles were independent among all pairs of loci (Fisher's Exact test, $P > 0.05$ in all cases). These results indicated that there was no pseudo-replication for heterozygosity estimates due to genotypic linkage disequilibrium. No conspicuous deviations of allele frequencies between the two sex types were observed, and heterozygosity was not related to sex type at any of the six loci (GLMs; sex: $P > 0.45$ in all cases). Estimates of population inbreeding coefficients did also not vary significantly between loci (ANOVA, $F_{5,36} = 0.29$, $P = 0.915$), indicating that there was no outlier locus with respect to selective neutrality.

Adult populations

The microsatellite allelic diversity in adult populations of *T. praecox* agg. was similar at contrasting altitudes, except for the study region Z where the alpine population showed lower genetic diversity than the subalpine population (ANOVA; altitude: $F_{1,4.5} = 0.43$, $P = 0.545$; region: $F_{3,3.9} = 1.18$, $P = 0.423$; altitude × region: $F_{3,15} = 2.86$, $P = 0.072$; Table 1). There was also no significant difference in the observed heterozygosity ($wMLH_O$) of flowering adult individuals of *T. praecox* agg. among altitudes (altitude: $F_{1,3} = 0.51$, $P = 0.526$; region: $F_{3,3} = 2.57$, $P = 0.229$; altitude × region: $F_{3,126} = 2.51$, $P = 0.062$), and the two sexual phenotypes did not significantly differ in $wMLH_O$ as well (sex: $F_{1,132} = 0.00$, $P = 0.999$; altitude × sex: $F_{1,132} = 1.74$, $P = 0.189$). Adult populations showed a weak tendency toward heterozygote excess as compared to Hardy-Weinberg expectations (Table 1), with similar estimates of population inbreeding coefficients found at contrasting altitudes, but the inbreeding coefficients tended to vary across regions (altitude: $F_{1,36} = 0.44$, $P = 0.509$; region: $F_{3,36} = 2.28$, $P = 0.096$). Furthermore, a comparison of pairwise genetic similarities between closely and distantly spaced individuals within populations revealed no evidence of a difference in the extent of genetic substructure among altitudes (altitude: $F_{1,3} = 0.36$, $P = 0.592$; distance: $F_{1,3} = 3.68$, $P = 0.151$; altitude × distance: $F_{1,3} = 0.04$, $P = 0.847$), but there was some variation in substructure among populations (altitude × distance × region: $F_{3,126} = 2.61$, $P = 0.054$; Table 1). Across populations, the degree of genetic substructure was positively correlated with the average spatial distance between plants (Spearman correlation: $r_s = 0.88$, $P = 0.004$; Table 1). Accordingly, average spatial distances between hermaphrodite individuals were also not significantly different at the contrasting altitudes, but there was significant variation among populations (altitude: $F_{1,3} = 0.76$, $P = 0.447$; region: $F_{3,3} = 0.69$, $P = 0.616$; altitude × region: $F_{3,72} = 3.76$, $P = 0.014$; Table 1). However, there was a significant effect of altitude on floral display size, with hermaphrodites at high altitude having fewer inflorescences than hermaphrodites at low altitude, and with significant variation among populations (altitude: $F_{1,3} = 15.93$, $P = 0.028$; region: $F_{3,3} = 1.00$, $P = 0.498$; altitude × region: $F_{3,334} = 3.81$, $P = 0.010$; Table 1).

Selfing rates

Rates of geitonogamous selfing in seedlings of open-pollinated individual hermaphrodites ranged from 0.00 to 0.86 (mean = 0.37 ±0.04 SE) among low altitude populations and from 0.00 to 1.00 (mean = 0.41 ±0.04 SE) among high altitude populations of *T. praecox* agg. Selfing rates did not generally differ between altitudes (altitude: $F_{1,6} = 0.36$, $P = 0.570$; population nested within altitude: $F_{6,73} = 1.91$, $P = 0.091$), but in one of the four study regions, S, the alpine population showed higher rates of selfing than the subalpine population (Fig. 1). The spatial arrangement and floral display size of potential outcross pollen donors significantly affected individual selfing rates, which decreased with increasing neighbourhood pollen availability at both low and high altitude sites (Table 1). For the high altitude, however, interaction plots (not shown) indicated that the effect of neighbourhood pollen availability on selfing rate was more pronounced within populations SH and ZH than within populations LH and PH. In contrast, a significant positive relationship between floral display size and individual selfing rate was only detected in the low altitude populations (Table 1). The apparent difference in the relationship between floral display size and selfing rate across altitudes was, however, attributable to few low altitude individuals exhibiting the largest floral display sizes, as revealed by an ANCOVA on a reduced set of individuals harbouring a maximum of 100 inflorescences (floral display size: $F_{1,58} = 0.01$, $P = 0.938$; altitude × floral display size: $F_{1,58} = 0.35$, $P = 0.558$). An additional exclusion of individuals experiencing especially low or high neighbourhood pollen availabilities finally revealed nearly identical estimates of pollinator-mediated selfing rates at both altitudes (low altitude: mean = 0.37 (±0.05 SE); high altitude: 0.39 (±0.03 SE); *t*-test: $P = 0.76$).

Costs of self-fertilisation

No significant costs of self-fertilisation were detected for early stages in the life cycle of *T. praecox* agg. under experimental conditions. The average seed set per fruit of hand-pollinated hermaphrodites was 2.57 (±0.06 SE) and, overall, not significantly affected by the cross treatment ($F_{1,1} = 0.00$, $P = 0.997$), but the treatment effect varied between the two study regions ($F_{1,25} = 8.38$, $P = 0.008$; relative performance of self- to cross-pollinated maternal half-sib families in population PH: mean = 1.05 ±0.06 SE; PL: 1.09 ±0.06; ZH: 0.95 ±0.04; ZL: 0.93 ±0.03). The average germination ratio of the crosses on hermaphrodites was 0.59 (± 0.03 SE), and germination was also not significantly affected by the two treatments ($F_{1,26} = 2.41$, $P = 0.132$; relative performance of self- to cross-pollinated maternal half-sib families in population PH: mean = 1.04 ±0.27 SE; PL: 0.97 ±0.04; ZH: 1.04 ±0.19; ZL: 0.93 ±0.11).

In contrast, the mean observed heterozygosity of selfed seedlings ($n = 213$) was significantly lower than the mean heterozygosity of adults (generation: $F_{1,3} = 195.99$, $P < 0.001$; Fig. 2), indicating significant lifetime costs of self-fertilisation in natural populations of *T. praecox* agg. The inferred costs of self-fertilisation varied slightly across regions (generation × region: $F_{3,486} = 2.79$, $P = 0.040$; Fig. 2), but there was no evidence of a

difference in the magnitude of these costs at contrasting altitudes (altitude × generation: $F_{1,486} = 0.16$, $P = 0.690$). The average loss of heterozygosity through self-fertilisation within families was 0.168, but the variance in heterozygosity of selfed offspring was large both within families (data not shown) and within populations (Fig. 2). The proportion of selfed offspring showing heterozygosity that was lower than the heterozygosities found among adults also varied across regions (logistic regression: likelihood ratio $\chi^2_3 = 11.95$, $P = 0.007$), whereas altitude did not generally affect this proportion, although, in study region P, an almost lacking overlap between the heterozygosities of selfed offspring and the heterozygosities of adults within the high altitude population contrasted with a considerable corresponding overlap within the low altitude population (altitude: $\chi^2_1 = 2.97$, $P = 0.085$; proportion within population LH: 0.48, LL: 0.53, PH: 0.92, PL: 0.52, SH: 0.80, SL: 0.80, ZH: 0.58, ZL: 0.50).

Outcrossed offspring

The mean heterozygosity of outcrossed seedlings ($n = 328$) from hermaphrodite seed-parents did not significantly differ from the mean heterozygosity of adult populations (generation: $F_{1,3} = 3.45$, $P = 0.160$; generation × region: $F_{3,600} = 1.71$, $P = 0.164$), and there was no significant variation in the difference between the two generations across altitudes (altitude × generation: $F_{1,600} = 0.15$, $P = 0.693$; Fig. 2). The proportion of outcrossed offspring showing heterozygosity that was lower than the heterozygosities found among adults varied across regions ($\chi^2_3 = 11.01$, $P = 0.012$), but there was also no significant effect of altitude ($\chi^2_1 = 1.53$, $P = 0.215$; proportion within population LH: 0.02, LL: 0.00, PH: 0.12, PL: 0.11, SH: 0.16, SL: 0.10, ZH: 0.12, ZL: 0.05).

Discussion

I chose a molecular genetic approach to investigate outcrossing advantage in gynodioecious *T. praecox* agg. at two contrasting altitudes in the Swiss Alps. My study is the first to use co-dominant microsatellite markers to estimate costs of self-fertilisation in natural populations of a tetraploid plant with polysomic inheritance and also among the first to explore impacts of elevation gradients on the realised mating system of a widely distributed mountain plant species. Evidently, my interpretations and conclusions critically depend on the reliability of the used microsatellite markers. The analysis of genetic variation at the six loci studied indicated that these microsatellite loci provided largely independent and apparently neutral genetic markers (Selkoe & Toonen, 2006). Moreover, the finding of similarly high allelic diversity in low altitude and in high altitude populations of *T. praecox* agg. confirmed that the marker-based estimates of heterozygosity and mating patterns were comparable across altitudes. Below I discuss my results in the context of nuclear-cytoplasmic gynodioecy, polyploidy and pollination biology.

In the present work, I did not investigate in detail a third component of the outcrossing advantage of females, namely biparental inbreeding (Sun & Ganders, 1988; Thompson &

Tarayre, 2000), and its relative importance in the two sexual phenotypes. However, variation in the albeit low proportions of outcrossed offspring (from hermaphrodites) which showed lower heterozygosities than those found in the corresponding adult populations may provide insight into the variation of biparental inbreeding among populations of *T. praecox* agg. Indeed, these proportions covaried with the extent of population genetic substructure (Spearman correlation: $r_s = 0.59$, $P = 0.119$), a major determinant of biparental inbreeding in plants (Sun & Ganders, 1988; Glaettli *et al.*, 2006). Neither population genetic substructure nor minor differences in heterozygosity between adults and outcrossed offspring from hermaphrodites were yet related to the altitudinal sex ratio variation in *T. praecox* agg. (Fig. 2; Table 1). As population genetic substructure should affect biparental inbreeding in females in a similar way than in hermaphrodites, it thus appears rather unlikely that differences in the extent of biparental inbreeding among sexual phenotypes could cause the observed sex ratio variation in *T. praecox* agg. In the following, I therefore focus on two other components of outcrossing advantage in *T. praecox* agg., i.e. the cost and rate of self-fertilisation in hermaphrodites.

Costs of self-fertilisation

The primary interest to estimate costs of self-fertilisation in *T. praecox* agg. is in comparing their relative magnitude in populations from the two contrasting altitudes. The results were unambiguous. (1) There was no evidence of an interaction effect between cross treatment (selfing vs. outcrossing) and altitude on seed set and germination success, confirming that seedling-based estimates of the mating system and of inter-generation differences in heterozygosity can directly be compared between altitudes. (2) The difference in heterozygosity between adults and selfed seedlings was as pronounced at low altitudes as at high altitudes (Fig. 2), indicating that the strength of selection against selfed offspring is equal in subalpine and alpine populations of *T. praecox* agg. (Ritland, 1990). As a consequence, costs of self-fertilisation were not associated with the sex ratio variation among populations in *T. praecox* agg.

However, absolute estimates of the chance of selfed offspring to survive to reproduction under natural conditions are desirable in order to understand equilibrium sex ratios in *T. praecox* agg. (Bailey & McCauley, 2005; Landergott *et al.*, submitted). A realistic estimation of the costs of selfing would also need to take into account the offspring of females (Kohn & Biardi, 1995), and knowledge on the relative fecundity of the two sexual phenotypes and on maternal effects on survival rates would be required (Ashman, 1992; Delph & Mutikainen, 2003). Only considering the offspring from hermaphrodites underestimates the costs of selfing. However, the distributions of heterozygosities observed among the adults and among selfed and outcrossed offspring from hermaphrodites may nevertheless provide insight into the effective costs of self-fertilisation. Note that, although up to half of selfed offspring per population displayed values of heterozygosity overlapping with the heterozygosity range of the corresponding adults, most values of selfed offspring actually fell within the lower 25% quantile of adult heterozygosities (Fig. 2). If only selfed offspring were to contribute to the

lowermost heterozygosities in the next generation, one may estimate the probability of selfed offspring to reach reproductive maturity by multiplying (a) the probability of the heterozygosity of selfed offspring to overlap with the heterozygosity of adults with (b) the probability of heterozygosity of adult individuals to overlap with heterozygosities among selfed offspring. However, within the range of overlapping heterozygosity values between adults and selfed offspring, selfed offspring will also compete with outcrossed offspring, which will further reduce probability (b) according to the proportions of selfed and outcrossed offspring expected within this range of heterozygosities (adjusted for the population specific selfing rate). This calculation yielded estimates of the chance of selfed offspring to survive to reproduction that ranged from 0.002 in population PH to 0.084 in population ZH. These estimates are in agreement with the slight heterozygote excess detected in adult populations, and the results strongly suggest that selfed offspring are culled by selection and that essentially only outcrossed offspring will contribute to the next generation in natural populations of *T. praecox* agg.

The detected magnitude of the reduction in fitness of selfed offspring relative to outcrossed offspring, commonly referred to as inbreeding depression (Husband & Schemske, 1996), may be surprising for a polyploid species (Galloway *et al.*, 2003), which raises the question of the genetic basis of the deleterious effects of selfing in *T. praecox* agg. (Charlesworth & Charlesworth, 1999). The observed pattern of almost lacking costs of selfing in early stages of the life cycle, in combination with significant estimates of lifetime costs of selfing in *T. praecox* agg. may point to inbreeding depression attributable to mildly deleterious mutations (Husband & Schemske, 1996). High mutational load may indeed be maintained in gynodioecious populations showing effectively random mating that reduces opportunities for purging (Ramsey *et al.*, 2006b). However, given the high levels of heterozygosity observed in populations of *T. praecox* agg., one might expect inbreeding depression due to mildly deleterious alleles to be genetically buffered in a first generation of selfing in this autotetraploid species (Bever & Felber, 1992; Galloway *et al.*, 2003), suggesting that other mechanisms could be involved in the costs of selfing. In fact, using apparently neutral markers, heterozygosity-based estimates of costs of selfing may be affected by overdominant selection at unlinked fitness-determining loci (Charlesworth, 1991; Hansson & Westerberg, 2002). One theory of the maintenance of nuclear-cytoplasmic gynodioecy predicts overdominance at nuclear loci that restore the male function in hermaphrodite individuals (Bailey *et al.*, 2003), and there is indeed growing evidence for extraordinarily high levels of heterozygosity at restorer loci in hermaphrodites of gynodioecious species (Bailey & McCauley, 2005). Interestingly, this finding coincides with reports of a tendency toward excess heterozygosity in natural populations of several species with nuclear-cytoplasmic gynodioecy (this study; Wolff *et al.*, 1988; van Treuren *et al.*, 1993; Lopez-Pujol *et al.*, 2004; Ramsey *et al.*, 2006a), suggesting that the genetic basis of the outcrossing advantage under nuclear-cytoplasmic gynodioecy deserves further investigation. For the time being, I therefore hesitate to use my results from gynodioecious *T. praecox* agg. for drawing general conclusions on costs of selfing

in polyploid species. However, the finding of constantly high costs of self-fertilization implies that variation in the outcrossing advantage among populations of *T. praecox* agg. will be determined by variation in the rates of selfing.

Rates of geitonogamous selfing

Only in one out of the four study regions, I detected a substantial difference in selfing rates among the populations from contrasting altitudes. In this deviating region S, the high altitude population showed a higher selfing rate than the low altitude population (Fig. 1a), and the inferred outcrossing advantage of females was thus correlated with population sex ratio within this region (Table 1). The interpretation of this result requires an understanding of the proximate cause for the observed pattern: Does the variation in selfing rates among populations reflect spatial and temporal variation caused by annual fluctuations in pollinators (Aide, 1986), or is the variation in selfing rates attributable to consistent structural variation among populations in region S? In the absence of repeated estimates of selfing rates among years, an inspection of those factors that mainly impact on selfing rates of individual hermaphrodites is most useful for understanding the variation in selfing rates among populations.

Individual selfing rates in *T. praecox* agg. were prominently affected by local neighbourhood structure, i.e. by the spatial proximity and the floral display size of neighbouring hermaphrodites, regardless of altitude (Table 2). The distance and size of close-by flower patches are expected to affect the foraging behaviour of pollinators and the extent of geitonogamous pollination of a focal plant in multiple ways: a high 'neighbourhood pollen availability' will increase the rate of pollinator visitation, the probability of receiving visitors carrying conspecific outcross pollen and the probability of early departure of visitors (Goulson, 1999). Such effects of small-scale population density on individual selfing rates have previously been reported in gynodioecious *Salvia pratensis* (van Treuren *et al.*, 1993) and *T. vulgaris* (Brabant *et al.*, 1980), and individual selfing rates have been found to be highly consistent among years in the latter species (Valdeyron *et al.*, 1977). Structural variation in the study populations also provides a plausible explanation for the observed variation in selfing rates among populations of *T. praecox* agg.: the lowest mean selfing rate was found in population SL and corresponded to the highest mean neighbourhood pollen availability (Fig. 1a, b), caused by a higher-than-average floral display size of neighbours (Table 1). On the other hand, the highest mean selfing rate in population SH corresponded to the lowest estimate of neighbourhood pollen availability (Fig. 1a, b), attributable to higher-than-average spatial distances among hermaphro-dites (Table 1). Similarly, in population ZL, relatively high selfing rates were associated with high average spatial distances among hermaphrodites (Fig. 1a; Table 1). I therefore consider the altitudinal variation in selfing rates in region S to be attributable to structural characteristics of the sampled populations SH and SL.

Similarly, an apparent difference in the relationship between focal plant floral display and individual selfing rate among altitudes (Table 2) resulted from differences in plant size at the contrasting altitudes, rather than from differences in pollinator behaviour. In fact, a direct

effect of floral display size on the extent of geitonogamous selfing was only evident in the largest individuals of *T. praecox* agg., whereas no such effect was detected over a wide range of five to 100 inflorescences per individual, contrary to theoretical expectations and empirical findings in other plant species (de Jong *et al.*, 1993; Karron *et al.*, 2004; Brunet & Sweet, 2006). This negative result in the present study may simply be due to statistical error inherent in the small sample sizes used to estimate individual selfing rates. However, the pronounced relationship between selfing rates and local neighbourhood structure discussed above demonstrates the detectability of strong effects, facilitated by the high resolution power of the microsatellite markers used (Ivey & Wyatt, 1999). Thus, a potential effect of floral display size (up to 100 inflorescences) on rates of geitonogamous selfing should be distinctly weaker than the effect of local neighbourhood structure in natural populations of *T. praecox* agg. Interestingly, the effect of local neighbourhood structure on selfing rates may actually have mitigated the effect of floral display of focal plants, since the floral display size of focal plants was positively correlated with the floral display size of neighbouring individuals in *T. praecox* agg. (Pearson correlation coefficients averaged across populations, high altitude: $r = 0.44$, low altitude: $r = 0.43$). Therefore, non-adaptive costs of geitonogamous selfing may well be largely constant over a considerable range of floral display sizes in natural populations of *T. praecox* agg., and individuals at high altitudes mostly fitted to this range. The above scenario of context-dependent mating may shed new light on the evolution of floral display size in plants (Barrett, 2003; Fabbro & Körner, 2004; Karron *et al.*, 2004). Comprehensive studies focussing on the effects of local neighbourhood structure on geitonogamous selfing under natural conditions will therefore be most valuable.

Taken together, my data revealed no evidence of a pollinator-mediated change in the realised mating system in *T. praecox* agg. along subalpine to alpine elevation gradients. On the contrary, my findings demonstrate that efficient cross-fertilisation is possible in an alpine environment, thus complementing a growing list of studies that point to efficient pollination of alpine plants (Kearns & Inouye, 1994; Bingham & Orthner, 1998; Gugerli, 1998; Totland & Schulte-Herbruggen, 2003), and also indicating that a high level of generalisation in the pollination system may help to maintain outcrossing across pronounced ecological gradients (Waser *et al.*, 1996; Eckert, 2002). Moreover, mean population selfing rates were generally very similar at contrasting population sex ratios, suggesting that the negative effect of lower hermaphrodite frequency and reduced floral display size of outcross pollen donors on selfing rates at high altitudes (Fig. 1b) was counterbalanced by the negative effect of largest floral displays of focal plants found at low altitudes.

Outcrossing advantage and sex ratio variation

More than one third of the seeds of hermaphrodites are self-fertilised and will consequently fail to reach reproductive maturity in natural populations of *T. praecox* agg., as inferred from my marker-based analysis. This implies a substantial outcrossing advantage of females, as it has also been reported in other gynodioecious plant species (Kohn & Biardi, 1995; Sakai *et al.*,

1997; Medrano *et al.*, 2005; Ramsey *et al.*, 2006a), raising the question of the importance of inbreeding avoidance in the maintenance of the gynodioecious breeding system (Charlesworth, 1999; Webb, 1999). Testing the importance of inbreeding avoidance in species with nuclear-cytoplasmic gynodioecy yet represents a difficult task, because it requires to isolate the effect of selection to avoid inbreeding from multiple selective forces acting simultaneously on different components of this complex genetic and phenotypic polymorphism (Charlesworth, 1999; Jacobs & Wade, 2003). Relating variation in outcrossing advantage to intra-specific sex ratio variation provides a useful empirical approach to estimate the significance of inbreeding avoidance (Charlesworth, 1999; Webb, 1999; Medrano *et al.*, 2005). In *T. praecox* agg., a comparison of adult and offspring sex ratios has shown that environment-dependent selection affecting the relative seed fitness of the two sex types may indeed govern the sex ratio variation along elevation gradients, with the expected relative seed fitness of hermaphrodites being reduced at higher altitudes (Landergott *et al.*, submitted). However, my present study provided no support for the hypothesis that variation in ecological conditions directly affected the outcrossing advantage of females in relation to sex ratio variation in *T. praecox* agg., because cross-fertilisation of hermaphrodites was equally efficient at high altitudes as at low altitudes (except in region S; see above) and because selection against selfed offspring was equally severe at low altitudes as at high altitudes. I therefore conclude that selection to avoid inbreeding is not the driving force that generally maintains the sex ratio variation among the study populations. The magnitude of the detected outcrossing advantage, and the observation that it appeared to be balanced across populations, nevertheless highlights the importance of incorporating outcrossing advantage as a component of the relative seed fitness of the two sexual phenotypes in future attempts to explain equilibrium sex ratios in *T. praecox* agg.

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Table 1 Summary statistics for genetic attributes, plant size and density in eight adult populations of *Thymus praecox* agg. from low and high altitude sites in four regions of the Swiss Alps. Sex ratios refer to the proportion of hermaphrodites per population (Landergott *et al.*, submitted). Estimates of allelic richness (A_{26}), expected heterozygositiy (H_E) and population inbreeding coefficients (F_{IS}, with upper 95% confidence limits) were averaged across six microsatellite loci. The genetic similarity between individuals and their nearest neighbour was divided by their similarity with a more remote plant and similarity ratios were averaged (± SE) to estimate the degree of genetic population substructure. Estimates of floral display size were determined from ln-transformed data; back-transformed means and 95% confidence limits are given. The average spatial distance (± SE) between hermaphrodites was calculated from average distances between focal plants and hermaphrodites among their six nearest neighbours.

		Females and hermaphrodites					Hermaphrodites	
	Altitude	Sex	Allelic	Hardy-Weinberg expectations		Population substructure:	Floral display	Average spatial
Population	[m a.s.l.]	ratio	richness	Multilocus H_E	F_{IS} (upper 95% CL)	genetic similarity ratio	size (95% CLs)	distance [m]
LH	2180	0.57	18.9	0.821	-0.003 (0.014)	1.03 (±0.10)	19 (15, 25)	0.83 (±0.06)
LL	1690	0.63	18.2	0.824	-0.012 (0.008)	0.95 (±0.07)	32 (24, 42)	0.85 (±0.08)
PH	2410	0.46	17.2	0.843	-0.025 (-0.006)	1.33 (±0.12)	16 (12, 20)	0.88 (±0.14)
PL	1715	0.62	17.7	0.805	-0.024 (-0.008)	1.03 (±0.09)	23 (18, 30)	0.78 (±0.06)
SH	2175	0.52	18.5	0.863	-0.001 (0.010)	1.70 (±0.24)	16 (12, 22)	0.99 (±0.24)
SL	1155	0.61	18.4	0.850	-0.006 (0.014)	1.35 (±0.25)	56 (43, 74)	0.95 (±0.07)
ZH	2010	0.48	18.3	0.831	-0.017 (0.008)	1.21 (±0.15)	16 (12, 21)	0.86 (±0.06)
ZL	1460	0.67	21.0	0.850	-0.021 (-0.008)	1.65 (±0.17)	39 (28, 53)	1.27 (±0.13)

Table 2 Effects of neighbourhood pollen availability and floral display size on individual selfing rates of hermaphrodites in *Thymus praecox* agg., analysed separately for four low altitude and four high altitude populations by means of general linear models (see materials and methods for model selection). The model for the low altitude populations explains 35.3% of the variation in selfing rates, the model for the high altitude populations 30.4%. Standardised coefficients are shown for the two continuous predictor variables.

Model	Source	Std *ß*	d.f.	MS	*F*	*P*
Low altitude	Neighbourhood pollen availability	-0.54	1	0.65	14.63	<0.001
	Floral display size	0.45	1	0.44	9.88	0.003
	Residual		35	0.04		
High altitude	Neighbourhood pollen availability	-0.43	1	0.19	4.33	0.045
	Floral display size	0.14	1	0.02	0.48	0.495
	Population		3	0.02	0.27	0.843
	Neighbourhood pollen availability × population		3	0.08	1.92	0.145
	Residual		33	0.04		

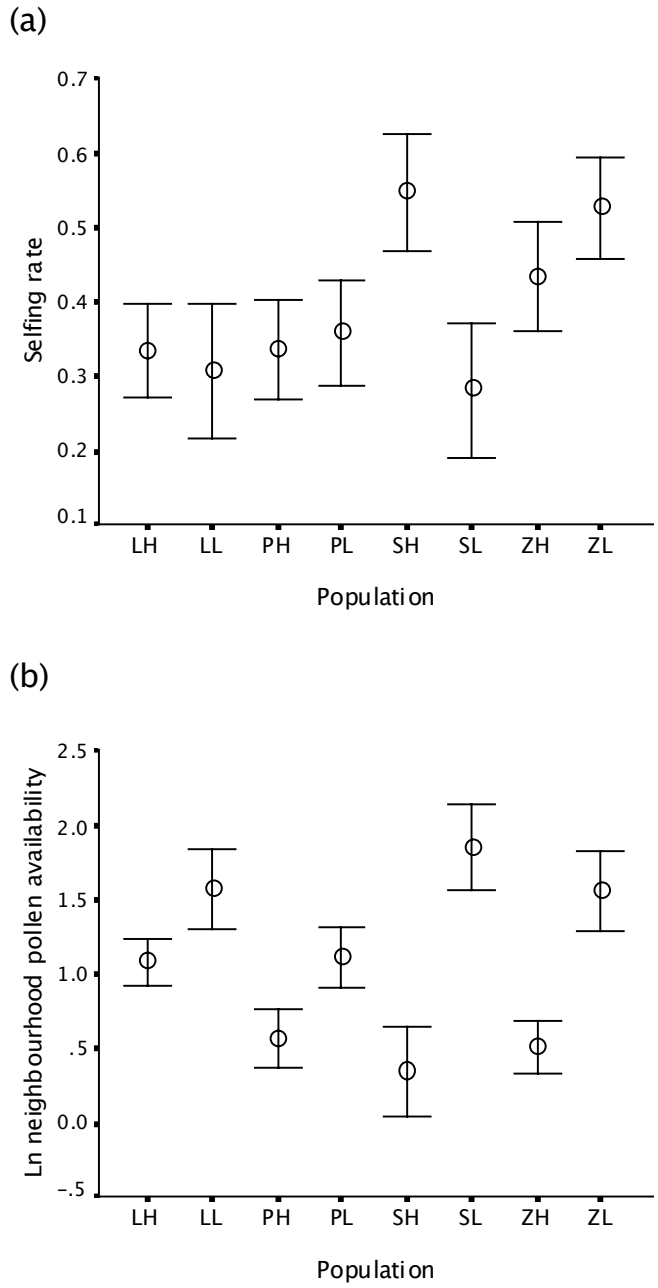

Figure 1 Estimates of selfing rates in hermaphrodites (a) and of neighbourhood pollen availability (b) in four high altitude and four low altitude populations of *Thymus praecox* agg. from four regions of the Swiss Alps (L, P, S, Z = four regions; H, L = high and low altitude, respectively). Data are means ± SE (*n* = 9-11 hermaphrodite seed-parents per population); neighbourhood pollen availabilities were ln-transformed.

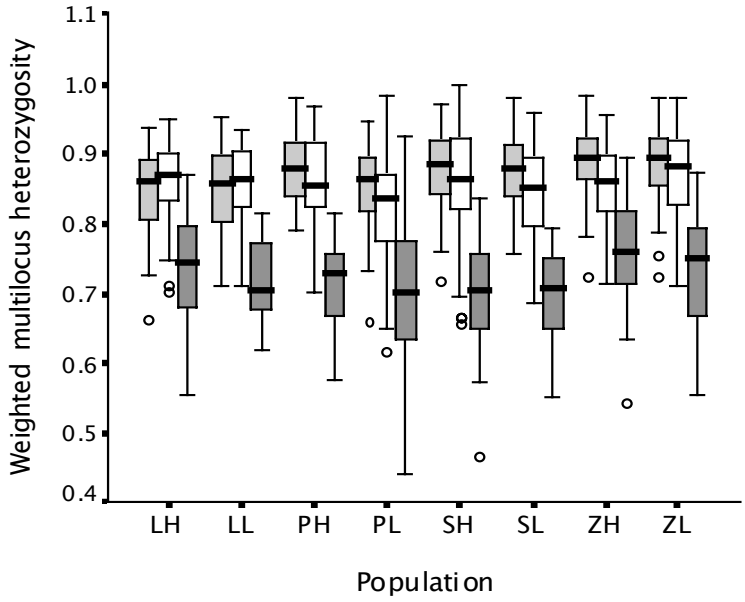

Figure 2 Box-plots showing medians and distributions of observed weighted multilocus heterozygosities ($wMLH_O$) for adult individuals (light grey), outcrossed seedlings (white) and selfed seedlings (dark grey) of eight populations of *Thymus praecox* agg. from four regions (L, P, S, Z) and two contrasting altitudes (H=high, L=low) in the Swiss Alps. (Medians were nearly identical to back-transformed means of arcsine-transformed data.)

Summary

The coexistence of females and hermaphrodites (gynodioecy), occurring in several groups of flowering plants, provides a useful system for studying the evolution and maintenance of sexual and genetic polymorphism. A persistent polymorphism for both nuclear and cytoplasmic sex-determining genes sheds light on nuclear-cytoplasmic interactions and co-evolution: cytoplasmic genes cause male-sterility in females; nuclear genes act to restore male function in hermaphrodites. In addition, the sexual dimorphism gives insight into principle properties of plant breeding systems, such as the relevance of outcrossing. Sex ratio variation among natural populations of gynodioecious plant species provides a key to understand the evolutionary processes and the environmental and genetic factors involved in the maintenance of nuclear-cytoplasmic sex systems. Mountain ranges, characterised by steep ecological gradients, offer a “natural experiment” to study the potential effects of environmental factors on the breeding system of plant species. In my thesis, I explored proximate causes of sex ratio variation in tetraploid, gynodioecious Mountain Thyme (*Thymus praecox* agg.) across elevation gradients in the Swiss Alps.

In **Chapter 1**, I examined sex ratio variation in relation to the genetic diversity and spatial dynamics of sex-determining genes. The proportion of hermaphrodites in 30 adult populations of *T. praecox* significantly decreased with increasing altitude. This correlation between population sex ratio and altitude, replicated in different study regions, suggested that the study system was indeed affected by environmental factors, i.e. that sex ratio variation among populations was not governed by limit cycles in a dynamic equilibrium of sex-determining genes. Furthermore, reciprocal transplant experiments demonstrated that the expression of the two sex morphs was stable and independent of temperature and altitude in *T. praecox*. Why are then more females found at higher altitudes? Under nuclear-cytoplasmic gynodioecy, hermaphrodites typically carry a cytoplasmic male-sterility factor in combination with appropriate nuclear genes restoring male function. Therefore, is a lack of appropriate restorers of male function, as it may result from stochastic evolutionary processes or isolation by distance, responsible for the reduced frequency of hermaphrodites at higher altitudes in *T. praecox*? I analysed progeny sex ratios from open-pollination and from controlled crosses within and among populations to estimate the genetic diversity at sex-determining loci in low and high altitude populations of *T. praecox*. Progeny sex ratios from natural populations did not generally differ between altitudes, and hermaphrodite fathers from contrasting altitudes showed similar restoration abilities in among population crosses. Hence, the sex ratio variation among adult populations was not attributable to variation in the genetic diversity of sex-determining loci in *T. praecox*. Moreover, within population crosses yielded higher proportions of hermaphrodite offspring than among population crosses, indicating that sex-determining genes were subjected to selective processes (local adaptation between nuclear and cytoplasmic sex-determining genes or selection against mismatched restorers due to costs of restoration),

irrespective of altitude. These results suggest that the evolutionary history of the study populations allows the detection of signatures of selective forces acting on this gynodioecious system, and ecological factors affecting the relative seed fitness and survival rate of the two sexual phenotypes could govern the sex ratio variation along elevation gradients in *T. praecox*.

In many gynodioecious species, female advantage in seed fitness results from outcrossing advantage over partially self-fertilised hermaphrodites. Thus, if hermaphrodites suffer from higher rates of selfing at high altitudes, or if selection against inbred offspring (costs of selfing) is more severe at higher than at lower altitudes, outcrossing advantage of females may provide an explanation for the sex ratio variation in *T. praecox*. This hypothesis was attractive to pursue since it coincides with classical views on harsh alpine conditions that may provoke increased rates and costs of self-fertilisation at high altitudes. A test of these hypotheses needs estimations of the rates and the costs of selfing under natural conditions, which can be done using highly variable molecular genetic markers resolving differences in heterozygosity among individuals, since selfing reduces heterozygosity.

In **Chapter 2**, I provided a protocol for the development and validation of highly variable and fully informative (co-dominant) microsatellite markers in tetraploid species with polysomic inheritance. The polyploid nature of *T. praecox* caused two major technical obstacles. First, high genetic variation in microsatellite flanking-sequences – likely a result of the reticulate evolutionary history of the tetraploid study species – required extensive knowledge on sequence variation in order to avoid amplification failure due to mutations in primer sites (null alleles). Second, because up to four copies of an allele can occur at a given locus, polymerase chain reaction (PCR) conditions had to be optimised for accurate estimation of allele copy numbers from PCR product intensities. Mendelian segregation of the six studied microsatellite markers in controlled crosses finally confirmed the quantification of allele copy numbers in PCR. The developed microsatellite markers thus provide reliable estimates of heterozygosity in *T. praecox*. From a general point of view, this chapter points out access to fully informative PCR-based markers representing a powerful tool for molecular genetic analyses in polyploid species.

In **Chapter 3**, I examined the outcrossing advantage in relation to altitude and sex ratio variation in natural populations of *T. praecox*. I used six microsatellite markers to estimate two major components of the outcrossing advantage of females in subalpine and alpine populations replicated over four valleys from the Swiss Alps, namely the rates and costs of self-fertilisation in hermaphrodites. To explore the main factors affecting selfing rates, I recorded the floral display size (number of inflorescences) of focal hermaphrodites, their local neighbourhood pollen availability (spatial distance and floral display size of close-by hermaphrodites) and their individual selfing rate inferred from genetic analysis of open-pollinated offspring. Floral displays were significantly larger at low than at high altitude and, consequently, local neighbourhood pollen availability was lower in high than in low altitudinal populations. Selfing rates of individual hermaphrodites at both altitudes were significantly affected by local neighbourhood pollen availability, as expected in pollinator-mediated geitonogamous self-

fertilisation. In contrast, floral display size of focal hermaphrodites did not significantly affect the rate of geitonogamous selfing, except for a few very large individuals exclusively occurring at low altitudes. When only considering individuals with comparable floral display size and neighbourhood pollen availabilities, selfing rates were nearly identical at low and high altitudes, rejecting the hypothesis that alpine conditions promote pollinator-mediated selfing in *T. praecox*. Accordingly, variation in selfing rates among populations within altitude was well explained by variation in average local neighbourhood pollen availabilities among populations. Overall, population selfing rates were not significantly different between low and high altitudes (mean selfing rate across all populations = 0.39), indicating that the negative effect of reduced neighbourhood pollen availability on selfing rates at high altitude was counterbalanced by the increased selfing of especially large focal plants at low altitude. To examine the costs of self-fertilisation at contrasting altitudes, I compared heterozygosities of selfed offspring with heterozygosities of adult individuals. Average heterozygosity of selfed offspring was significantly lower than the heterozygosity of corresponding adults, but the magnitude of the inferred costs of selfing was independent of altitude. Moreover, the magnitude of the difference in heterozygosity between adults and selfed offspring and the finding of slight heterozygote excess in adult populations suggested that selfed offspring largely fails to reach reproductive maturity in natural populations of *T. praecox* at both altitudes. Taken together, cross-fertilisation of hermaphrodites was equally efficient at high altitudes as at low altitudes, and selection against selfed offspring was equally severe at low as at high altitudes. These results reject the hypothesis that the outcrossing advantage of females governs the variation in the relative seed fitness of the sexual phenotypes and, consequently, the sex ratio variation across altitudinal gradients in *T. praecox*.

In conclusion, neither the genetic diversity of sex-determining genes nor the outcrossing advantage of females did explain the observed sex ratio variation in gynodioecious *T. praecox* across subalpine to alpine elevation gradients. Furthermore, additional unpublished data revealed no evidence of a causal relationship between female fecundity advantage and female frequency in natural populations at contrasting altitudes. The failure of the above hypotheses to explain sex ratio variation in *T. praecox* may point to the importance of another, hitherto largely un-explored factor potentially governing sex ratio variation in gynodioecious plant species, namely the cost of restoration of male function. In particular, survival rates should be studied in relation to sexual phenotype, maternal sex and cost of restoration as caused by mismatched restorer alleles or inefficient sets of restorer alleles in *T. praecox*.

In addition, the present work gave surprising insight into geitonogamous pollination in relation to elevation, raising the more general question of whether efficient cross-fertilisation is rather the rule than the exception in alpine environments. Finally, the successful implementation of highly informative molecular genetic markers in tetraploid *T. praecox* should facilitate analysis of breeding system and population genetics in other polyploid plant species.

Zusammenfassung

Das Nebeneinander von Weibchen und Zwittern (Gynodiözie) in verschiedenen Gruppen der Blütenpflanzen bietet Gelegenheit, die Evolution von getrennten Geschlechtern und von genetischem Polymorphismus zu untersuchen. Die für die Aufrechterhaltung dieses Geschlechtsdimorphismus notwendige Aufrechterhaltung eines genetischen Polymorphismus von geschlechtsbestimmenden Genen gewährt Einblicke in Interaktionen und Co-Evolution zwischen Genen des Zellkerns (Nukleus) und Genen in Organellen des Zellplasmas (Cytoplasma): cytoplasmatische Gene unterdrücken die männliche Funktion (Weibchen), nukleäre Gene stellen die männliche Funktion wieder her (Zwitter; nukleo-cytplasmatische Gynodiözie). Ferner kann die Evolution von Gynodiözie auch Aufschluss geben über grundsätzliche Eigenschaften der Fortpflanzung bei Pflanzen, wie z.B. über die Bedeutung der Fremdbefruchtung. Variation in der relativen Häufigkeit der beiden Geschlechter zwischen gynodiözischen Populationen kann Hinweise geben auf die evolutiven Prozesse und auf die ökologischen und genetischen Faktoren, die das gynodiözische Fortpflanzungssystem erhalten. Gebirge bieten mit ihren ausgeprägten ökologischen Gradienten ein „Experiment der Natur“ zu möglichen Einflüssen von wechselnden Umweltbedingungen auf das Fortpflanzungssystem von Pflanzen. In meiner Dissertation habe ich Ursachen für die Variation der Geschlechterhäufigkeiten zwischen Populationen des gynodiözischen Bergthymians (*Thymus praecox* agg.) entlang von Höhengradienten in den Schweizer Alpen untersucht.

Das **1. Kapitel** behandelt den Zusammenhang zwischen Geschlechterhäufigkeiten und der genetischen Vielfalt und räumlichen Verteilung der geschlechtsbestimmenden Gene. Der Anteil von Zwittern in Populationen des Bergthymians nahm signifikant ab mit zunehmender Höhe über Meer. Diese Korrelation zwischen Geschlechterhäufigkeit und Höhenstufe, wiederholt in verschiedenen Alpentälern, wies darauf hin, dass das nukleo-cytoplasmatische gynodiözische System tatsächlich von Umweltfaktoren beeinflusst wurde. Reziproke Verpflanzungsexperimente haben gezeigt, dass die Ausprägung des Geschlechts einzelner Individuen des Bergthymians stabil war und demnach unabhängig von Höhenstufe oder Temperatur. Warum gab es also mehr Weibchen in höheren Lagen? Bei nukleo-cytoplasmatischer Gynodiözie sind die meisten Zwitter „wiederhergestellte Zwitter“, d.h. sie tragen ein cytoplasmatisches Sterilitätsgen in Kombination mit passenden nukleären Genen, welche die männliche Funktion wiederherstellen. Demnach könnte ein Mangel an passenden Wiederherstellungsgenen, verursacht beispielsweise durch zufällige genetische Drift, in höher gelegenen Populationen des Bergthymians zu erhöhter Weibchenhäufigkeit führen. Ich habe deshalb die Geschlechterhäufigkeiten in Nachkommen aus offener (natürlicher) Bestäubung und aus kontrollierten Kreuzungen innerhalb und zwischen Populationen verwendet, um die genetische Vielfalt der geschlechtsbestimmenden Gene in tief und hoch gelegenen Populationen zu schätzen. Die Geschlechterhäufigkeiten in den Nachkommen waren nicht signifikant verschieden zwischen hoch und tief gelegenen Populationen, und die Kreuzungen

wiesen ebenfalls auf ähnliche genetische Vielfalt der Wiederherstellungsene auf den beiden Höhenstufen hin. Die unterschiedlichen Häufigkeiten der beiden Geschlechter zwischen Erwachsenen-Populationen verschiedener Höhenstufen beruhten also nicht auf Unterschieden in der Vielfalt der geschlechtsbestimmenden Gene. Hingegen ergaben die Kreuzungen innerhalb der Populationen einen grösseren Anteil zwittriger Nachkommen als die Kreuzungen zwischen den Populationen, was darauf hinwies, dass die geschlechtsbestimmenden Gene natürlicher Selektion unterlagen (lokale Anpassung zwischen nukleären und cytoplasmatischen Genen oder Selektion gegen unpassende nukleäre Wiederherstellungsgene infolge „Kosten der Wiederherstellung der männlichen Funktion"). Zusammen liessen diese Resultate darauf schliessen, dass Umweltfaktoren, welche die relative Samen-Fitness oder die relativen Überlebensraten von Zwittern und Weibchen beeinflussen, die Variation der Geschlechterhäufigkeiten entlang des Höhengradienten erklären könnten.

Bei vielen gynodiözischen Arten ist ein Teil eines allgemein zu beobachtenden Weibchenvorteils in Bezug auf die Samen-Fitness (Beitrag von Nachkommen zur nächsten Generation via Samen) auf einen Fremdbefruchtungsvorteil der Weibchen gegenüber teilweise selbstbestäubten Zwittern zurückzuführen. Wenn also Zwitter in höheren Lagen vermehrt selbstbestäubt sind, oder wenn die Selektion gegen Nachkommen aus Inzucht (Nachteil der Selbstbefruchung) in höheren Lagen stärker ausgeprägt ist, könnte der Fremdbefruchtungsvorteil der Weibchen deren erhöhte Häufigkeit in höheren Lagen erklären. Diese Hypothese schien besonders naheliegend, weil sie mit klassischen Erwartungen von erhöhten Selbstbestäubungsraten (andere Insektengruppen oder anderes Verhalten der Blütenbesucher) und von einem ausgeprägteren Nachteil der Selbstbefruchtung unter unwirtlichen alpinen Lebensbedingungen übereinstimmte. Moderne molekulargenetische Methoden erlauben es, die Häufigkeit von Selbstbestäubung und den Nachteil der Selbstbefruchtung unter natürlichen Lebensbedingungen abzuschätzen. Dazu müssen die verwendeten molekulargenetischen Marker Unterschiede in der Heterozygotie verschiedener Individuen auflösen können.

Im **2. Kapitel** beschreibe ich die Entwicklung von sechs hochvariablen Mikrosatelliten-Markern für die Verwendung beim polyploiden Bergthymian (tetraploid, d.h. das Kerngenom wurde verdoppelt und liegt in vier statt wie üblich in zwei Kopien vor). Die Polyploidie des Bergthymians hat dabei zwei grössere technische Probleme verursacht: (1) Hohe genetische Vielfalt in den flankierenden Sequenzen der Microsatelliten-Loci – wohl das Resultat von Polyploidisierung und Hybridisierung in der evolutiven Geschichte der Art – erforderte umfangreiche Sequenzierarbeiten, um für die Amplifikation der Microsatelliten-Loci geeignete Startersequenzen zu finden. (2) Der Bergthymian zeigt ein polysomisches Vererbungsmuster, d.h. es können pro Locus ein bis vier identische Allele vorkommen. Die für die Amplifikation der Mikrosatelliten-Marker verwendete Polymerase-Kettenreaktion (PCR) musste also so optimiert werden, dass von der Menge der Amplifikationsprodukte die Anzahl Allele bestimmt werden konnte. Eine Test-Analyse von Nachkommen aus kontrollierten Kreuzungen hat schliesslich bestätigt, dass die Quantifizierung der Allelkopien mittels PCR korrekt war, d.h.

dass die Mikrosatelliten-Marker den Heterozygotiegrad beim Bergthymian zuverlässig wiederzugeben vermochten. Dieses Kapitel zeigt neue Möglichkeiten auf für die Verwendung von hochauflösenden molekulargenetischen Markern in genetischen Untersuchungen von polyploiden Pflanzen (darunter viele Kulturpflanzen).

Im **3. Kapitel** untersuche ich mögliche Auswirkungen der Höhenstufe auf den Fremdbefruchtungsvorteil in natürlichen Populationen des Bergthymians. Ich verwendete die Mikrosatelliten-Marker, um die zwei Hauptbestandteile des Fremdbefruchtungsvorteils von Weibchen, nämlich die Selbstbestäubungsrate und den Nachteil der Selbstbefruchtung bei Zwittern, in subalpinen und alpinen Populationen aus vier verschiedenen Alpentälern zu schätzen. Dazu bestimmte ich für zufällig ausgewählte Zwitterpflanzen und deren Nachkommen aus offener Bestäubung die Mikrosatelliten-Genotypen und ermittelte daraus den Anteil der aus Selbstbestäubung stammenden Nachkommen, also die Selbstbestäubungsrate der Zwitter. Die Selbstbestäubungsrate einzelner Zwitter hing signifikant von der Zwitterpflanzen-Dichte in ihrer unmittelbaren Umgebung ab; je mehr andere Zwitter vorhanden waren, desto niedriger war die ermittelte Selbstbestäubungsrate. Dies entsprach den Erwartungen unter von Insekten verursachter geitonogamer Selbstbestäubung (zwischen verschiedenen Blüten der gleichen Pflanze), und der gefundene Effekt war gleich auf beiden Höhenstufen. Dagegen zeigte die Pflanzengrösse (Anzahl Blütenstände) selbst keinen signifikanten Einfluss auf die Selbstbestäubungsrate, abgesehen von erhöhten Selbstbestäubungsraten besonders grosser Zwitter, die ausschliesslich in tief gelegenen Populationen vorkamen. Nach Einbezug dieser strukturellen Unterschiede zwischen subalpinen und alpinen Populationen war die geschätzte Selbstbestäubungsrate auf beiden Höhenstufen praktisch identisch. Dies zeigte, dass die Blütenbesucher an alpinen Standorten beim Bergthymian keine erhöhten Selbstbestäubungsraten verursachten, verglichen mit den subalpinen Standorten. Ferner wogen sich die negativen Effekte von geringerer Zwitterdichte in höheren Lagen und jene von besonders grossen Pflanzen in tieferen Lagen im Mittel gegenseitig auf, so dass auch die mittleren Selbstbestäubungsraten der Populationen (Durchschnitt = 0.39) nicht signifikant verschieden waren zwischen den Höhenstufen. Selbstbefruchtung reduziert den Heterozygotiegrad. Die Heterozygotie der Nachkommen aus Selbstbefruchtung war signifikant niedriger als die Heterozygotie der Erwachsenen-Populationen, unabhängig von der Höhenstufe. Tatsächlich wies die Grösse des Unterschieds zwischen der Heterozygotie von erwachsenen Pflanzen und der Heterozygotie von Nachkommen aus Selbstbefruchtung darauf hin, dass sowohl in den tief als auch in den hoch gelegenen Populationen des Bergthymians kaum Nachkommen aus Selbstbefruchtung zur Fortpflanzung gelangen, d.h. der Nachteil der Selbstbefruchtung war gravierend. Alles in allem war also die Kreuzbestäubung der Zwitter in alpinen Populationen gleich effektiv wie in den subalpinen Populationen, und der Nachteil der Selbstbefruchtung war ebenso schwerwiegend in den subalpinen wie in den alpinen Populationen des Bergthymians. Diese Resultate widerlegten die Hypothese, dass der Fremdbefruchtungsvorteil der Weibchen ausschlaggebend war für deren erhöhte Häufigkeit in hoch gelegenen Populationen des Bergthymians.

Schliesslich konnten weder die genetische Vielfalt der geschlechtsbestimmenden Gene noch der Fremdbefruchtungsvorteil der Weibchen beim gynodiözischen Bergthymian die beobachtete Variation der Geschlechterhäufigkeiten entlang von Höhengradienten erklären. Unveröffentlichte Daten zum Fekunditätsvorteil der Weibchen haben ferner auch keinen Zusammenhang zwischen Fekunditätsvorteil und Häufigkeit der Weibchen ergeben. Dass die obigen Hypothesen die Variation der Geschlechterhäufigkeiten nicht zu erklären vermochten, könnte auf die Bedeutung eines weiteren, bisher noch kaum untersuchten Faktors hinweisen, nämlich der sogenannten „Kosten der Wiederherstellung der männlichen Funktion“ bei Zwittern. Insbesondere sollten beim Bergthymian die Überlebensraten untersucht werden im Hinblick auf den Einfluss des Geschlechts, des Geschlechts der Mutterpflanze und der Kosten der Wiederherstellung der männlichen Funktion infolge unpassender oder ineffizienter Kombinationen von geschlechtsbestimmenden Genen.

Zusätzlich ergab die vorliegende Arbeit aber auch überraschende Resultate bezüglich Selbstbestäubung und Höhenlage, welche die allgemeinere Frage aufwerfen, ob effektive Kreuzbestäubung im alpinen Lebensraum gar eher die Regel als die Ausnahme darstellt. Die erfolgreiche Anwendung von hochauflösenden molekulargenetischen Markern beim tetraploiden Bergthymian dürfte schliesslich auch die Populationsgenetik und Untersuchungen des Fortpflanzungssystems anderer polyploider Pflanzenarten erleichtern.

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Lebenslauf

Name	LANDERGOTT
Vorname	Urs
Geburtsdatum	13. Januar 1975
Heimatort und Kanton	Winterthur ZH

Ausbildung

Mittelschule	Kantonsschule Rychenberg Winterthur 1994 Abschluss Matura Typus B
Hochschulstudium	Universität Zürich 1995 bis 1997 Grundstudium der Biologie 1997 bis 2000 Diplomstudium mit Hauptfach Systematische Botanik, Nebenfach Zoologie und weiterem Nebenfach Geobotanik (ETH Zürich)
Diplomfach	Botanik
Titel der Diplomarbeit	Populationsgeschichte und genetische Variabilität des seltenen Kammfarns (*Dryopteris cristata*)
Doktorat	2001 bis 2006 Anstellung als Doktorand am Institut für Systematische Botanik der Universität Zürich 2006 Stipendium der Janggen-Pöhn Stiftung, Gast an der Eidg. Forschungsanstalt für Wald, Schnee und Landschaft WSL